SepsiTest™-UMD

Pathogen DNA Extraction and PCR Analysis

Direct universal PCR detection of
bacteria and fungi in:

Body fluids

(*ascites, BAL, blood,* CSF*, plasma, pleural fluid, pus, synovial fluid*)

Swabs

(mouth, nasopharynx, wounds, bones)

Tissues

(*abscesses*, *biopsies, heart valves, prostheses*)

Internal extraction control assay, positive PCR control and sequencing primers included

CE IVD

– For *in-vitro* diagnostic use –

molzym
reveal DNA

©2017-2023 Molzym, all rights reserved

Version 03

Date of first release: 05/2017
Last update: 01/2023

Contents page

Kit Information **4**
Kit Contents – *SepsiTest™-UMD* **4**
Symbols **5**
Storage and Stability **6**
Intended Use and Indication **7**
Contraindication 7
Product Use Limitations 7
Apparatuses and Consumables to be Supplied by the User **8**
Safety Information **10**
Hazard and Precautionary Statements 10

Introduction **12**
System Description **12**
Test Principle **12**

Controls and Validation **15**
Controls **15**
Internal Extraction Control 15
Sample Controls 15
Positive Sample Control 15
Negative Sample Control 15
PCR Controls 16
Positive PCR Control 16
Negative PCR Control 16
Validation **17**
Broad-range Primers 17
List of Strains Tested for Extraction 17
Analytical Specificity 17
Analytical Sensitivity 17
Clinical Evaluation 17

Avoidance of DNA Contamination **19**

Part 1: Pre-Analytics **20**
Sample Collection **21**
Isolation of Pathogen DNA **21**
Protocols **22**
1A) How to Start 22
1B) Arrangements and Pre-Treatment of Samples 23
i) Body fluids 23
(ascites, BAL, blood, CSF, plasma, pleural fluid, pus, synovial fluids) 23
ii) Swabs (mouth, nasopharynx, wounds, bones) 23
iii) Tissue samples (abscesses, biopsies, heart valves, prostheses) 23
1C) DNA Isolation Protocol 24

Part 2: Analytics **26**
Description of the Assays **27**
Packaging, Storage and Handling **27**
Quality Control and Specifications **28**
PCR Detection and Identification of Pathogens by Sequencing **28**
PCR Detection ***28***
2A) How to Start 29
Avoidance of DNA Contamination *29*
Storage of the PCR Reagents after Handling *29*
Places where Handlings are performed *29*

PCR Assays *30*
2B) Setup of the Assays 31
2C) PCR Thermocycling 32
2D) Detection by Agarose Gel Electrophoresis 32
2E) Guidance to the Interpretation of PCR Results 33
Identification of Pathogens by Sequencing of Amplicons 34
2F) Purification of Amplicons 34
2G) Sequencing 35
2H) SepsiTest™-BLAST Analysis for Strain Identification 35
Addendum: Real-Time PCR Protocols 36
Patents/Disclaimer 36
General Requirements 36
Real-Time PCR Thermocycling and Detection by Melting Curve Analysis 37
Guidance to the Interpretation of Real-Time PCR Results 40
Supplementary Information 45
Troubleshooting 45
References 47
Tradenames 50
Technical Support 50
Order Information 51
Contact 52

Kit Information

Kit Contents – *SepsiTest™-UMD*

DNA Isolation	24 rxn (U-010-024)	48 rxn (U-010-048)
Kit 1 - Buffers & Consumables (store at +18 to +25°C)		
A) Extraction Buffers, in DNA-free bags		
CM	6x 1.0 ml	12x 1.0 ml
DB1	6x 1.0 ml	12x 1.0 ml
RS	6x [4x 1.0 ml]	12x [4x 1.0 ml]
RL	6x 0.32 ml	12x 0.32 ml
RP	6x 0.6 ml	12x 0.6 ml
CS	6x 1.0 ml	12x 1.0 ml
AB	6x 1.0 ml	12x 1.0 ml
WB	6x 1.6 ml	12x 1.6 ml
WS	6x 1.6 ml	12x 1.6 ml
ES	6x 0.4 ml	12x 0.4 ml
B) Consumables, in DNA-free bags		
ST - Sample tubes, 2.0 ml	6x 4	12x 4
C) Consumables, in DNA-free bags		
SC – Spin columns in 2.0 ml *Collection tubes*	6x 4	12x 4
CT - Collection tubes, 2.0 ml	6x 8	12x 8
ET – Elution tubes, 1.5 ml	6x 4	12x 4
D) Tissue Pre-treatment Buffers, in white boxes		
TSB	1x 25 ml	2x [1x 25 ml]
PKB	4x 1.2 ml	2x [4x 1.2 ml]
E) Sample Dilution Buffer, in white boxes		
SU	2x [12x 1.0 ml]	4x [12x 1.0 ml]
DNA Isolation	**24 rxn**	**48 rxn**
Kit 2 – Enzymes & Reagents (store at -15 to -25°C), in white boxes		
Enzyme K, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]
MolDNase B, solution	2x [3x 0.04 ml]	4x [3x 0.04 ml]
BugLysis, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]
ß-mercaptoethanol, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]
Proteinase K, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]
Internal Extraction Control Unit (store at -15 to -25°C), in bags (in Kit 2)		
Control DNA	2x [3x 0.01 ml]	4x [3x 0.01 ml]
PCR Detection and Identification	**24 rxn**	**48 rxn**
Kit 3 - PCR Reagents (store at -15 to -25°C), in white boxes		
MA Bac, Mastermix Assay Bacteria, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]
MA Yeasts, Mastermix Assay Yeasts, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]
MA Control, Mastermix Assay Control, 2.5x conc.	1x 0.36 ml	2x [1x 0.36 ml]
MolTaq 16S/18S	3x 0.05 ml	2x [3x 0.05 ml]
H_2O - DNA-free PCR-grade water	3x 0.75 ml	2x [3x 0.75 ml]

Continued on next page

DS - DNA staining solution, 10x conc.	2x 0.30 ml	2x [2x 0.30 ml]
Kit 4 - PCR Controls & Detection Reagents (store at -15 to -25°C)		
A) Detection Reagents (white box)		
LS - Gel loading solution, 6x conc.	1x 0.4 ml	1x 0.4 ml
SM - DNA size marker	1x 0.24 ml	1x 0.24 ml
SeqGP16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml
SeqGN16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml
SeqYeast18 - Sequencing primer (fungi)	1x 0.1 ml	2x 0.1 ml
B) Positive PCR Control (bag)		
DNA Standard P1, for PCR positive control runs	1x 0.3 ml	1x 0.3 ml
DNA dilution buffer (for P1)	1x 25 ml	2x 25 ml
Consumables PCR Detection & Identification (store at +18 to +25°C), in Kit 1		
MT - Mastermix tubes, 1.5 ml (for Kit 3)	2x 50	3x 50
Manuals (in Kit 1)	**24 rxn**	**48 rxn**
Manual	1x	1x
Short manual sheets	4x	4x

Symbols

Symbols used in labelling and in section 'Hazard and Precautionary Statements' (pages 10 to 11).

For *in-vitro* diagnostic use


This product fulfils the requirements of the European Directive 98/79 EC for *in-vitro* diagnostic medical devices.


Content of the package


Manufactured by


Temperature limitation (store at)

Consult instructions for use

Batch code

Catalogue number


Use by


Flammable


Corrosive


Toxicity


Irritant


Health hazard


Environmentally Hazardous

Storage and Stability

Guarantee for full performance of reagents and buffers is given through the expiration date printed on the label at the outer box, if the packed material is undamaged upon arrival and the reagents are unopened. Guarantee for full performance of ***SepsiTest™-UMD*** as specified in this manual is only valid if storage conditions are followed (Tab. 1).

Once opened, the vials have to be used as specified by the protocol.

The DNA Isolation unit, Kits 1 and 2 and the Internal Extraction Control Unit (Kit 2), is provided in vials containing reagents to perform 4 sample extractions to minimise the risk of carry-over contamination.

Kit 1 (Buffers & Consumables): Buffers and consumables of the DNA Isolation unit (packages A to E) must be stored dry in the dark and at room temperature (+18 to +25°C). Opened packages of vials/ consumables must be stored at room temperature (+18 to +25°C) for 4 days. The opened buffer *TSB* must be stored at room temperature (+18 to +25°C) in a dark, DNA-free place to the expiry date of the Kit. Opened vial of the *RP* buffer (Kit 1) including the *Control DNA* (Internal Extraction Control Unit) must be stored at room temperature (+18 to +25°C) for 4 days. Do not use the prepared *RP* buffer longer than 4 days.

Kit 2 (Enzymes & Reagents): Please take care that the vials of the DNA Isolation unit have to be stored at -15 to -25°C upon delivery.

Internal Extraction Control Unit (Kit 2): Please take care that the vials of the Internal Extraction Control Unit have to be stored at -15 to -25°C upon delivery.

Kits 3 (PCR Reagents) and 4A (Detection Reagents): Please take care that the vials of the PCR Detection unit (Kits 3 and 4A) have to be stored at -15 to -25°C upon delivery. It is important to note that the DNA staining solution (*DS*, Kit 3) and the DNA size marker (*SM*, Kit 4A) are sensitive to light and must be stored dark during handling and storage. ***Do not freeze again*** (*DS*, *SM* and *LS*) and store at +4 to +12°C for further use. After use, the mastermixes and H_2O must be stored in the refrigerator (+4 to +12°C) for further use at the same day but must be replaced to -15 to -25°C for longer storage.

Kit 4B (Positive PCR Control): Please take care that the components of the PCR Detection unit (Kit 4B) have to be stored at -15 to -25°C upon delivery. The reagents of Kit 4B must be stored at +4 to +12°C after the first handling.

Tab. 1: Storage of the ***SepsiTest™-UMD*** components (*exp. date: expiry date of the kit).

Components	Storage Temperature	Working Temperature	Storage & Stabilitiy after the Usage Temperature	Days (dark)*
Kit 1 - Buffers & Consumables:				
CM, DB1, RS, RL, RP, CS, AB, WS, ES	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	4
Consumables (*ST, SC, CT, ET*)	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	4
TSB	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
PKB	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	4
SU	+18 to + 25°C	+18 to + 25°C	single-use, no further storage	
Kit 2 - Enzymes & Reagents:				
Enzyme K, MolDNase B, BugLysis, ß-mercaptoethanol, Proteinase K	-15 to -25°C	-15 to -25°C	-15 to -25°C	exp. date
Internal Extraction Control Unit (in Kit 2):				
Control DNA	-15 to -25°C	+18 to + 25°C	+18 to + 25°C in buffer RP	4
Kit 3 - PCR Reagents:				
MA Bac, MA Yeasts, MA Control	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	1 (thereafter freeze)
			-15 to -25°C	exp. date
MolTaq 16S/18S	-15 to -25°C	-15 to -25°C	-15 to -25°C	exp. date
H2O	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	1 (thereafter freeze)
			-15 to -25°C	exp. date
DS (*DNA Staining Solution*)	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
Kit 4A - Detection Reagents:				
LS	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SM	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SeqGP16, SeqGN16, SeqYeast18	-15 to -25°C	+18 to + 25°C	-15 to -25°C	exp. date
Kit 4B - Positive PCR Control:				
P1 DNA Standard	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
P2 (prepare freshly for the PCR)	1:500 dilution of P1	+18 to + 25°C	+4 to + 12°C	1
DNA Dilution Buffer	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
Consumables PCR Detection & Identification, in Kit 1:				
MT - Mastermix tubes, 1.5 ml (for Kit 3)	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date

Intended Use and Indication

SepsiTest™-UMD is a kit for the detection of microbial DNA (bacterial/fungal). It is a set of reagents to detect the presence and identify bacteria and fungi in primary-sterile specimens. *SepsiTest™-UMD* is intended for body fluids, swabs and for tissues. The validated specimens are determined under 1B) Arrangements and Pre-Treatment of Samples

The kit is for laboratory use (professional users).

SepsiTest™-UMD is a kit for sample analysis of patients with suspected bacterial or fungal infection.

Contraindication

SepsiTest™-UMD reagent kit is not intended to be used as in vitro diagnostic test for the detection and identification of any specific pathogen. The results of ***SepsiTest™-UMD*** are not used as the sole basis for diagnosis, treatment, or other patient management decisions. ***SepsiTest™-UMD*** is not indicated for pathogens with safety level S3 and S4. An exemplary selection is listed in Tab. 2.

Tab. 2: Contraindication of ***SepsiTest™-UMD*** for pathogens with safety level S3 and S4 (exemplary selection).

Bacillus cereus biovar anthracis	*Mycobacterium microti*
Coxiella burnetii	*Mycobacterium pinnipedii*
Brucella abortus (B. melitensis biovar abortus)	*Mycobacterium tuberculosis (Mycobacterium tuberculosis subsp. tuberculosis)*
Brucella canis (B. melitensis biovar canis)	*Mycobacterium ulcerans*
Brucella inopinata	*Orientia tsutsugamushi (Rickettsia tsutsugamushi)*
Brucella melitensis (B. melitensis biovar melitensis)	*Rickettsia africae*
Brucella neotomae (B. melitensis biovar neotomae)	*Rickettsia akari*
Brucella ovis (B. melitensis biovar ovis)	*Rickettsia australis*
Brucella suis (B. melitensis biovar suis)	*Rickettsia conorii*
Burkholderia mallei (Pseudomonas mallei)	*Rickettsia heilongjiangensis*
Burkholderia pseudomallei (Pseudomonas pseudomallei)	*Rickettsia japonica*
Chlamydia psittaci (Chlamydophila psittaci)	*Rickettsia prowazekii*
Coxiella burnetii	*Rickettsia rickettsii*
Escherichia coli (enterohemorrhagic (EHEC) Strains O157:H7 or O103)	*Rickettsia sibirica*
Francisella tularensis subsp. tularensis	*Rickettsia typhi*
Mycobacterium africanum	*Salmonella Typhi*
Mycobacterium bovis	*Shigella dysenteriae*
Mycobacterium caprae (Mycobacterium tuberculosis subsp. caprae)	*Yersinia pestis*
Mycobacterium leprae	

Product Use Limitations

Usage of ***SepsiTest™-UMD*** reagents for clinical diagnostic tests requires validation of the in vitro diagnostic test procedure!

Whole blood samples must be collected and stabilized using either EDTA or citrate.

Sequencing results must be validated by a clinician to exclude false positive results originating from contaminations or clinically not relevant microorganisms.

SepsiTest™-UMD is not intended for frozen and thawed specimen materials. Not for other specimens than mentioned above.

Cells must be intact for reliable results. This requires specimens not to be stored in solutions, which induce cell lysis.

Transport media including charcoal medium and Amies should be avoided as they hold a risk of a strong inhibition of the amplification, which causes an invalid result. Inhibition would be detected by the extraction control. Test procedures must always be run including the extraction control and control assay provided with this kit.

Apparatuses and Consumables to be Supplied by the User

The following equipment, consumables and reagents not supplied with this kit are recommended to be used with ***SepsiTest™-UMD***.
Do not transfer supplies (e.g., pipettes, microcentrifuges, vortexer, racks) and disposable material as specified by the handlings below from one working place to another.

Sample preparation:

- 1x thermomixer (2.0 ml tubes), e.g., Eppendorf comfort, Eppendorf, Germany
- 1x cooling rack for 1.5 ml tubes (-15 to -25°C)
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 1x bench top microcentrifuge (≥12,000x*g*), e.g., miniSpin, Eppendorf, Germany
- 1x UV Class II biological safety cabinet
- Sample positive control (run control):
  BioBall® MultiShot 550 KBE, bioMérieux, Germany
  - BioBall® MultiShot *Candida albicans* NCPF 3179 (56003)
  - BioBall® MultiShot *Escherichia coli* NCTC 12923 (56006)
  - BioBall® MultiShot *Staphylococcus aureus* NCTC 10788 (56009)
- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany
- Sample racks
- Sterile forceps (only tissue protocol)
- Sterile support, e.g., Petri dish (only tissue protocol)
- Sterile scalpel or sterile preparation scissors (only tissue protocol)

PCR amplification:

- 1x UV workstation, e.g., GuardOne® Werkbank, Starlab, Germany
- 1x low speed mini-centrifuge (≤2000x*g*) e.g., MiniFuge, VWR, Darmstadt, Germany
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 2x cooling racks for 1.5 ml tubes (-15 to -25°C)
- 3x cooling racks for 0.2 ml PCR tubes (-15 to -25°C)
- PCR cycler, e.g., Mastercycler®, Eppendorf, Germany); other cyclers have to be validated by using positive PCR controls P1 and P2 according to the instructions (pages 29 to 32)
  Optional: Real-Time PCR instruments are validated (page 36 to 41)
- 2-3x sets of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany

Agarose gel electrophoresis analysis:

- Pre-cast gels (2 %) unstained, e.g., Reliant® Gel System, Lonza, USA; alternatively prepare a 2 % (w/v) agarose gel (e.g., LE agarose, Biozym, Germany) in 1x TAE buffer
- 1 electrophoresis chamber (15 x 34 cm, 1.5 l buffer volume capacity)
- Running buffer TAE (50x concentrated), e.g., Biozym, Germany
- An electrophoresis chamber with the following characters: 15 x 34 cm, buffer volume capacity: 1.5 litres
- An electrophoresis power supply (300 V, 500 mA), e.g., Consort E835, Sigma-Aldrich, USA
- A gel documentation system, e.g., system from Herolab, Germany
- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany

Sequencing:

- A DNA sequencing apparatus, e.g., DNA Analyzer ABI 3730XL®, ABI Prism310®
- Purification of amplicons, Qiagen, QIAquick® PCR Purification Kit (28104)
- Sequencing, e.g., BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA (optional: use an overnight sequencing service, e.g., Eurofins Genomics, Germany)
- Column for removal of dye terminators prior to sequencing (use of your internal validated material)
- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany

Plastic Consumables and reagents:

- Pipette tips (with aerosol filter), Biosphere® plus, Sarstedt, Germany
  - 10 µl type Eppendorf (70.1114.210)
  - 100 µl type Eppendorf (70.760.212)
  - 300 µl type Eppendorf (70.3040.255)
  - 1000 µl type Eppendorf (70.3050.255)
- 1.5 ml micro tubes, Biosphere® plus, Sarstedt, Germany (72.706.200)
  - For the preparation of the positive PCR control P2
  - For gel electrophoresis
- PCR tubes, e.g., PCR strip of 4, 200 µl, Biosphere® plus, Sarstedt, Germany (72.990)
- DNA decontamination, e.g., DNA/RNA-ExitusPlus™, AppliChem, Germany (A7089,0100)
- Surface decontamination, e.g., Meliseptol® New Formula (rapid disinfectant, ethanol containing), B. Braun, Germany (19758)
- Sterile disposables
  - Lab coat, e.g., VWR, Germany
  - Gloves, e.g., Kimberly-Clark, Germany
  - Sleeves, e.g., Cardinal Health, Ireland
  - Bouffant Covers, e.g., VWR, Germany
  - Hygiene mask, e.g., VWR, Germany
  - Overshoes, e.g., hygi, Germany
- Waste containers for plastics and liquid waste, autoclavable, for each working place

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable sleeve covers, disposable gloves and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDS) which are available on request.

CAUTION: Never add hypochlorite (bleach) or acidic solutions directly to the sample-preparation waste.

Buffers *CM* and *CS* contain guanidine hydrochloride and guanidinium thiocyanate, respectively, which can form highly reactive compounds and toxic gases when combined with hypochlorite or other acidic solutions. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 70 % (v/v) ethanol. This kit is to be used only by skilled personnel trained for handling infectious material and molecular biological methods. To avoid false analytic results by DNA contamination of reagents and infection of the user by infectious agents during handling, always wear sterile protective gloves, sterile disposable sleeve covers, a lab coat, sterile protective goggles and disposable overshoes. Work in a Class II biological safety cabinet irradiated with UV before starting according to the instruction manual of the manufacturer. The UV lamp must be switched off during working. Follow the instructions of the manufacturer for maintenance of the workstation. Dispose potentially infectious material and the waste of the sample preparation (part 1, page 20 to 25) according to the national directive of the health organisation (e.g., in Germany: Vollzugshilfe zur Entsorgung von Abfällen aus Einrichtungen des Gesundheitsdienstes, 2021).
Separate *Material Safety Data Sheets* are available on request. Molzym reserves the right to alter or modify the product to improve the performance of the kit.

Hazard and Precautionary Statements

Buffer *CM*

Contains guanidine hydrochloride (>10 %):
Acute toxicity (oral) and irritating (eyes and skin).

Warning

Hazard and precautionary statements*(page 11):
H302-H315-H319; P301+P312-P302+P352-P305+P351+P338

ß-mercaptoethanol

Contains 2-mercaptoethanol (100 %, CAS no. 60-24-2):
Acute toxicity (oral, inhalation, skin), irritation (skin), eye damage, skin sensitization, reproductive toxicity, specific target organ toxicity and hazardous to aquatic environment (acute and chronic).

Danger

Hazard and precautionary statements*(page 11):
H301+H331-H310-H315-H317-H318-H361d-H373-H410;
P273-P280-P301+P310-P302+P352+P310-P304+P340+P310-P305+P351+P338

Proteinase K / *Enzyme K*

Contains *Proteinase K* (≥ 1 %):
Respiratory sensitization and skin sensitization.

Danger

Hazard and precautionary statements*(page 11):
H317-H334; P280-P302+P352-P333+P313-P363

Buffer *RP* / Buffer *PKB*

Contains sodium dodecyl sulfate (< 10 %):
Acute toxicity (oral, inhalation), irritation (skin and eye).

Warning

Hazard and precautionary statements*:
H302-H315-H319-H332; P280-P301+P312-P304+P340+P312-P305+P351+P338

Buffer *CS*

Contains guanidinium thiocyanate (> 10 %): **Acute toxicity (oral, skin), skin sensitization, eye damage and hazardous to aquatic environment (chronic).**

Danger

Hazard and precautionary statements*:
H302-H312-H314-H318-H412-EUH032; P280-P303+P361+P353-P305+P351+P338-P310-P362+P364

Buffer *AB* / Buffer *WB*

Contains 2-propanol (*AB* > 40 % and *WB* ≥ 40 %): **Flammable liquids and irritating (eyes)**.

Danger

Hazard and precautionary statements*:
H225-H319-H336; P210-P233-P305+P351+P338

Buffer *WS*

Contains ethanol (> 50 %): **Flammable liquids and irritating (eyes)**.

Danger

Hazard and precautionary statements*:
H225-H319; P210-P233-P305+P351+P338

Emergency information (24-hours service)

For emergency medical information, please contact the regional poison center in your country.

* **H225:** Highly flammable liquid and vapour; **H302:** Harmful if swallowed; **H310:** Fatal in contact with skin; **H312:** Harmful in contact with skin; **H314:** Causes severe skin burns and eye damage; **H315:** Causes skin irritation; **H317:** May cause an allergic skin reaction; **H318:** Causes serious eye damage; **H319:** Causes serious eye irritation; **H332:** Harmful if inhaled; **H334:** May cause allergy or asthma symptoms or breathing difficulties if inhaled; **H336:** May cause drowsiness or dizziness; **H361d:** Suspected of damaging the unborn child; **H373:** May cause damage to organs (liver, heart) through prolonged or repeated exposure if swallowed; **H301+H331:** Toxic if swallowed or if inhaled; **H410:** Very toxic to aquatic life with long lasting effects; **H412**: Harmful to aquatic life with long lasting effects; **EUH032**: Contact with acids liberates very toxic gas.
P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.; **P233:** Keep container tightly closed; **P273:** Avoid release to the environment; **P280:** Wear protective gloves/protective clothing/eye protection/face protection; **P310:** Immediately call a POISON CENTER/doctor; **P363:** Wash contaminated clothing before reuse; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER or doctor; **P301+P312:** IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell; **P302+P352:** IF ON SKIN: Wash with plenty of water; **P302+P352+P310:** IF ON SKIN: Wash with plenty of water. Immediately call a POISON CENTER/doctor; **P303+P361+P353:** IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower]; **P304+P340+P310:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER/doctor; **P304+P340+P312:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/doctor if you feel unwell; **P305+P351+P338:** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing; **P333+P313:** If skin irritation or rash occurs: Get medical advice/attention; **P362+P364:** Take off contaminated clothing and wash it before reuse.

Introduction

System Description

This kit is marked according to European directive 98/79/EC **for *in-vitro* diagnostic use**.

SepsiTest™-UMD is a kit for the detection of bacterial and/or fungal DNA in body fluids, swabs and tissues. In analogy to the recording of a metabolite in microbiological culture detection of pathogens, ***SepsiTest™-UMD*** enables the detection of bacterial and fungal pathogens on a molecular level by the amplification of target sequences of rRNA genes. ***SepsiTest™-UMD*** can identify pathogens up to days earlier than culture and in patients who are negative with culture (Kühn et al. 2011; Orszag et al. 2013).

Molzym's technology of degradation of human DNA and isolation of pathogen target DNA from human samples is combined with universal rDNA PCR assays providing a high quality, straight forward kit for molecular pathogen detection.

By the enzymatic degradation of human DNA, pure microbial DNA is provided to the assays, minimising false results from unspecific primer binding. Furthermore, the high quality of all reagents of the kit guarantees tolerable reagent-borne false positive signalling (≤3 %) under the precautions of the avoidance of DNA contamination.

SepsiTest™-UMD allows the detection of essentially all bacterial and fungal pathogens, including both culturable and non-culturable strains. This is due to the amplification of 16S rDNA (bacteria) and 18S rDNA sequences (fungi) using universal primers. Sequence analysis of amplicons using primers provided with this kit is a confirmation of PCR results and allows the identification of strains detected in samples by BLAST analysis (e.g. www.sepsitest-blast.com).

Test Principle

In its concept, ***SepsiTest™-UMD*** is a means of molecular analysis of a broad-range of clinical specimens for the presence of pathogens. The system combines new solutions for sample preparation and PCR analysis of clinical specimens, in particular EDTA-stabilised whole blood samples, other body fluids, swabs and tissues (Fig. 2, page 14). The procedure includes DNA extraction of samples and PCR or Real-Time PCR analysis using primers targeting conserved regions of the 16S and 18S rRNA genes of bacteria and fungi, respectively. Amplicons are detected by agarose gel electrophoresis or melting curve analysis.

SepsiTest™-UMD is based on two basic steps (tissue is additionally pre-treated to release pathogens from biofilms):
i) Pathogens are enriched from the sample after the degradation of the human DNA and microbial DNA is purified by removal of PCR inhibitors.
ii) The eluate is analysed by universal rDNA PCR for pathogen DNA. Sequence analysis of amplicons together with BLAST search leads to the identification of pathogens.

Part 1: Pre-Analytics
Molzym has developed a technology enabling the enrichment and isolation of pure bacterial and fungal DNA from body fluids, tissues and swab samples for PCR analysis. The procedure comprises protocols for the pre-treatment of samples, including the degradation of DNA from lysed human cells, followed by the broad-range lysis of potentially present Gram-negative and Gram-positive bacteria as well as fungi.

The procedure in more detail (Fig. 1). A chaotropic buffer is added to the sample which selectively lyses the human cells, and the nucleic acids released are degraded by added *MolDNase*. Tissue samples are partially digested by a protease treatment before adding the chaotropic lysis buffer. After enrichment by centrifugation, pathogens are treated by a reagent, *BugLysis*, which degrades the cell walls of bacteria and fungi. Protein degradation by *Proteinase K* and protein denaturation by a chaotropic buffer finalise the extraction protocol. Finally, pathogen DNA is isolated by a bind-wash-elute procedure using Molzym's CCT technology which enables the recovery of femtogram to picogram amounts of DNA in a 100 µl eluate.

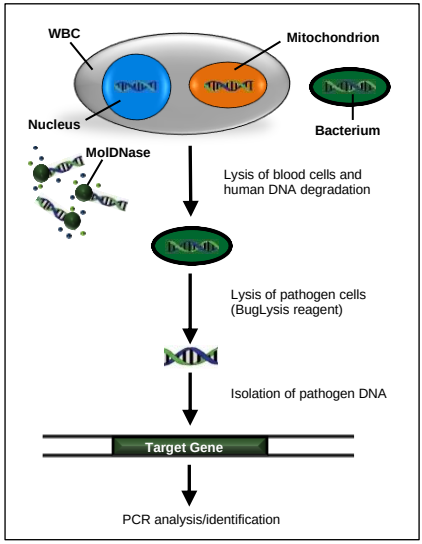


Fig. 1 The principle of testing for bacterial and fungal DNA in samples by ***SepsiTest™-UMD*** (blood as an example).

Part 2(a): PCR Analytics

DNA eluates (part 1, page 12) are used for broad-range 16S and 18S rDNA PCR analysis. Two assays are supplied, assay *MA Bac* (bacteria) and assay *MA Yeasts* (fungi) enabling the sensitive detection of pathogens. A protocol for the detection of amplicons by agarose gel electrophoresis is supplied. Real-Time PCR is another option. For testing the function of the extraction process and performance of the PCR assay regarding PCR inhibition by DNA extracts, a control DNA and control assay (*MA Control*) is included in the kit.

Part 2(b): Pathogen Identification by Sequence Analysis

A protocol for sequencing of amplicons is supplied in order to identify detected pathogens. The procedure includes a short protocol for amplicon purification and another for sequencing of amplicons. Primers for sequencing are supplied with this kit.

After sample extraction, at the first step negative or positive results are obtained by PCR or Real-Time PCR analysis, indicating the absence or presence of bacterial and fungal target sequences in the sample (Fig. 2). If positive, the second step of the analysis encompasses sequence analysis of amplicons. Sequencing is a way of confirming a positive PCR result

and of gaining information on the identity of an organism detected. Therefore, sequence analysis should always follow PCR detection of amplicons. Sequencing of amplicons from assay *MA Bac* (bacteria) employs primers, *SeqGN16* and *SeqGP16*.
SeqGN16 targets mainly Gram-negative bacteria and including few gram-positive bacteria. The excluding few gram-negative species will detect by *SeqGP16*.
SeqGP16 targets mainly Gram-positive bacteria and including few gram-negative species. The excluding species will be detected by *SeqGN16*. The primers are not strictly discriminative. The exceptions of the sequencing primers see in table on https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf.
By using both sequencing primers in separate reactions, mixed strains of the two groups can be resolved.
Sequencing of amplicons from the fungal assay, *MA Yeasts*, is performed using *SeqYeast18*.
Bacterial taxa are identified with the help of the free online tool, SepsiTest™-BLAST (www.sepsitest-blast.com). There are three potential outcomes of the sequencing analysis:
i) In case of readable sequences (Fig. 2, A, readable; below), the BLAST online search results in the identification of strains at the species (99 to 100 % sequence identity) or genus level (>97 % sequence identity), depending on the quality of the read.
Please note: For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST http://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid.
ii) If SepsiTest™-BLAST analysis indicates poor quality, overlapping sequences may be the reason as a result of more than one target sequence present in the reaction (Fig. 2, B, not readable); in such cases a service is available for identification of bacteria in mixed sequences (Pathogenomix, www.ripseq.com).
iii) If the amount of amplicon is too low for a sequencing reaction (Fig. 2, C, no read), the result is considered negative (below the limit of sequencing detection). In this case, another analysis of a new sample should be performed, if possible.

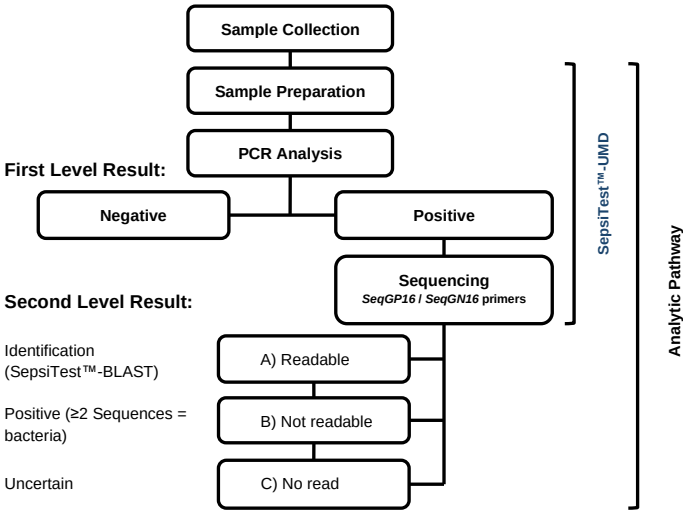


Fig. 2: Detection of microorganisms in samples using ***SepsiTest™-UMD*** and sequencing analysis. The analytic pathway includes the detection and identification of bacteria and fungi (only bacteria shown).

Controls and Validation

Controls

A series of controls should be routinely performed to test the performance of the kit. Below a list of controls is given and commented. More information on the exact procedures for running controls are given in the respective sections.

Internal Extraction Control

The Internal Extraction Control must be used for each sample and must be added to the extraction process in order to validate the extraction of DNA.

The *Control DNA* (Internal Extraction Control Unit in Kit 2) is a DNA template to be used as a process control to monitor DNA extraction from samples.

The kit supplies an assay (*MA Control*, Kit 3) to which an aliquot of the sample extract (including the *Control DNA*) is added. Generation of an amplicon indicates the correct function of the DNA extraction and purification process. Also, the absence of PCR inhibitors are indicated.

Sample Controls

Positive Sample Control

This control reflects the performance of the lysis and DNA extraction procedure from microorganisms and should be performed at least once per setup. There are two ways proposed to perform a positive sample control:

i) Negative samples (buffer *SU*, Kit 1) are spiked with 100 to 1000 cfu/ml of cultured Gram-negative (e.g., *E. coli*) or Gram-positive (e.g., *S. aureus*) and fungal pathogen (e.g., *C. albicans*), respectively, and run through the extraction protocol followed by analysis as described in this kit.

ii) The extraction is performed using a commercial standard. Molzym has evaluated BioBall® MultiShot 550 KBE (bioMérieux, Germany).

Negative Sample Control

This test should be run together with the positive sample control to test for potential cross-contamination during sample extraction. For this, a negative sample (buffer *SU*, Kit 1) is used and run through the extraction and detection protocols of this kit.

PCR Controls

Positive PCR Control

This test includes a definite number of target sequence copies to make sure that the assay is performing as specified. The *DNA Standard P1* (Kit 4B) comprises a mixture of DNAs extracted from *Bacillus subtilis* and *Saccharomyces cerevisiae*.
The set of controls comprises of a high (P1) and low (P2) standard DNA for Mastermix Assay Bacteria (*MA Bac*) and Mastermix Assay Yeasts (*MA Yeasts*). The high concentrated DNA standard (P1) is supplied with this kit and has a concentration of approx. 1.0 ng/µl (*MA Bac*) and approx. 0.1 ng/µl (*MA Yeasts*). Using this standard DNA indicates the functioning of the assays. The low concentrated DNA standard (P2) is diluted from P1 to approx. 2.0 pg/µl (*MA Bac*) and approx. 0.2 pg/µl (*MA Yeasts*) and constitutes a multiple of the lower limit of detection being a test for the sensitivity of the assays. Positive PCR controls P1 and P2 have to be performed with each set of analyses, i.e., with *MA Bac* and *MA Yeasts*.

Prepare the positive PCR control at a place where DNA is handled. Thaw DNA Standard P1 and DNA dilution buffer (Kit 4B). Vortex the P1 vial and pulse centrifuge. Pipette 998 µl of DNA dilution buffer in a 1.5 ml sterile polypropylene tube, add 2 µl DNA Standard P1 and vortex to mix. Always prepare P2 freshly for each series of PCRs. ***Do not re-use***, because dilute DNA solutions tend to be unstable.

Negative PCR Control

This setup contains all reagents except that supplied DNA-free water is added instead of eluate (target DNA). The control is meant to detect any exogenous DNA coming in as carry-over or handling contamination during running parallel tests and pipetting of reagents.

Validation

Broad-range Primers

The broad-range binding of the primers to universal sites of the 16S and 18S rRNA genes was analysed with a sequence alignment algorithm, allowing 1 mismatch (excluding terminal sites). As a result, more than 345 species are detectable, among which more than 200 species have been sequence-identified in clinical evaluations so far (Tab. 4, page 18).

List of Strains Tested for Extraction

SepsiTest™-UMD contains a reagent, *BugLysis*, for the degradation of cell walls of Gram-positive and Gram-negative bacteria, and fungi. The reagent has been evaluated with the following clinical strains, using Real-Time PCR for analysis (T_m analysis):

Gram-positive bacteria: *Bacillus cereus, B. subtilis, Corynebacterium diphteriae, Enterococcus faecalis, E. faecium, Lactobacillus sp., Micrococcus luteus, Mycobacterium bovis* (BCG), *Mycobacterium phlei, Staphylococcus aureus, S. carnosus, S. epidermidis, Streptococcus agalactiae* (Sero-Group B), *S. mutans*, *S. oralis, S. pneumoniae, S. pyogenes* (Sero-Group A), streptococci (Sero-Group G).

Gram-negative bacteria: *Acinetobacter baumannii*, *Escherichia coli, Enterobacter aerogenes, Serratia marcescens, Klebsiella pneumoniae, Neisseria meningitis, N. subflava, Porphyromonas gingivalis, Proteus mirabilis, P. vulgaris, Pseudomonas aeruginosa, P. stutzeri, Stenotrophomonas maltophilia.*

Fungi: *Aspergillus fumigatus, Candida albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Cryptococcus neoformans*.

Analytical Specificity

SepsiTest™-UMD includes two assays, one for the general detection of bacteria (*MA Bac*) and another for the detection of fungi (*MA Yeasts*). Experiments including yeast DNA in assay *MA Bac* and bacterial DNA in assay *MA Yeasts* gave no indication of cross reactivity of the primer pairs with the unspecific DNA. Cross reactivity was shown for bacterial primers used in assay *MA Bac* with a large excess of human DNA (see also Mühl et al. 2010). This problem is addressed by the pre-analytical treatment of samples to deplete human DNA (‘Test Principle’, pages 12 to 14).

Analytical Sensitivity

Molzym’s sample pre-treatment and DNA isolation constitutes the optimal solution for high sensitivity PCR and Real-Time PCR analysis of DNA from pathogenic bacteria and fungi. By this combination, for instance, *S. aureus* can be detected at 20 cfu/ml blood by the universal PCR assay. For other strains, see Tab. 3 (page 18) and Mühl et al. (2010). Spiking experiments using negative samples and serial dilutions of cultured strains of clinical isolates showed the detection limits indicated in Tab. 3, page 18.

Clinical Evaluation

A broad spectrum of Gram-negative, Gram-positive, and fungal organisms were identified in clinical samples using ***SepsiTest™-UMD*** (Tab. 4, page 18).

Tab. 3: Analytical sensitivity of ***SepsiTest™-UMD***.
Minimum titre resulting in positive results from 3 repeated extractions of fluid samples spiked with strains. Analysis: Real-Time PCR (5 µl eluate/assay; Assays: *MA Bac* and *MA Yeasts*) with melting curve analysis.

Strain	cfu/ml detected
Gram-positive bacteria	
Enterococcus faecalis	20
Staphylococcus aureus	20
Staphylococcus epidermidis	20
Streptococcus pneumoniae	75
Gram-negative bacteria	
Escherichia coli	40
Klebsiella pneumoniae	50
Moraxella catarrhalis	50
Pseudomonas aeruginosa	80
Fungi	
Candida albicans	10
Candida glabrata	10

Tab. 4: Extract of microorganisms identified in clinical evaluations

Gram-negative bacteria	*Helicobacter pylori*	*Alloiococcus otitis*	*Nocardia* spp.
Achromobacter xylosoxidans	*Kingella* spp.	*Anaerococcus* spp.	*Paenibacillus* spp.
Acidovorax spp.	*Klebsiella* spp.	*Atopobium* spp.	*Parvimonas micra*
Acinetobacter spp.	*Kerstersia* spp.	*Bacillus* spp.	*Peptoniphilus* spp.
Aeromonas veronii	*Kluyvera cryocrescens*	*Bifidobacterium* spp.	*Peptostreptococcus* spp.
Afipia broomeae	*Lautropia mirabilis*	*Brevibacterium* spp.	*Propionibacterium* spp.
Aggregatibacter aphorophilus	*Legionella pneumophila*	*Carnobacterium* spp.	*Rhodococcus* spp.
Anaerotruncus colihominis	*Leptotrichia* spp.	*Clostridium* spp.	*Rothia* spp.
Bacteroides spp.	*Massilia* spp.	*Coprococcus catus*	*Staphylococcus* spp.
Bartonella quintana	*Methylobacterium* spp.	*Corynebacterium* spp.	*Streptococcus* spp.
Bordetella spp.	*Moraxella* spp.	*Dermabacter hominis*	*Tropheryma whippleii*
Borrelia garinii	*Morganella morganii*	*Dietzia* spp.	*Tsukamurella* spp.
Bosea spp.	*Neisseria* spp.	*Dolosigranulum pigrum*	*Ureaplasma urealyticum*
Brucella spp.	*Pantoea agglomerans*	*Eggerthella lenta*	*Vagococcus* spp.
Burkholderia spp.	*Paracoccus* spp.	*Enterococcus* spp.	*Wolbachia* spp.
Campylobacter spp.	*Pasteurella* spp.	*Eremococcus coleocola*	
Candidatus Neoehrlichia mikurensis	*Porphyromonas* spp.	*Eubacterium* spp.	**Fungi**
Capnocytophaga spp.	*Prevotella* spp.	*Facklamia* spp.	*Aspergillus* spp.
Chryseobacterium indologenes	*Proteus* spp.	*Finegoldia magna*	*Candida* spp.
Citrobacter freundii	*Providencia stuartii*	Gardnerella vaginalis	*Cladosporium cladosporioides*
Cloacibacterium normanense	*Pseudomonas* spp.	*Gemella* spp.	*Cryptococcus* spp.
Comamonas testosteroni	*Ralstonia* spp.	*Gordonia* spp.	*Didymella exitialis*
Coxiella burnetii	*Raoultella planticola*	*Granulicatella adiacens*	*Davidiella tassiana*
Cronobacter sakazakii	*Rickettsia typhi*	*Janibacter* spp.	*Fusarium* spp.
Curvibacter spp.	*Serratia marcescens*	*Kocuria* spp.	*Issatchenkia orientalis*
Delftia spp.	*Shigella* spp.	*Lactobacillus* spp.	*Malassezia* spp.
Dialister spp.	*Stenotrophomonas maltophilia*	*Lactococcus* spp.	*Pseudallescheria boydii*
Elizabethkingia meningoseptica	*Veillonella* spp.	*Leifsonia* spp.	*Saccharomyces cerevisiae*
Enhydrobacter aerosaccus	*Weeksella* spp.	*Listeria monocytogenes*	*Schizophyllum radiatum*
Enterobacter spp.	*Yersinia* spp.	*Microbacterium* spp.	*Sporobolomyces* spp.
Escherichia spp.	**Gram-positive bacteria**	*Micrococcus* spp.	
Fusobacterium spp.	*Abiotrophia* spp.	*Mogibacterium timidum*	**Protist**
Haemophilus spp.	*Actinomyces* spp.	*Mycobacterium* spp.	*Plasmodium* spp.
Hafnia alvei	*Aerococcus* spp.	*Mycoplasma* spp.	

Upon request, Molzym can provide the full list of “Microorganisms found in clinical and other specimens by sequencing” including Gram-positive and Gram-negative bacteria, fungi and protists down to species level and is constantly updated.

Avoidance of DNA Contamination

Care should be taken to avoid DNA contamination from exogenous sources. This includes the complete pathway from sample collection to analysis. Also, it is important to minimise cross-contamination from sample to sample. For guidance see Roth et al. (2001) and Espy et al. (2006). A short summary of precautions is given below:

- **Guidelines**:
  The guidelines of the national health organisations, e.g., Robert-Koch-Institute (Germany), for sample collection, including sterilisation of the skin should be followed.

- **Decontamination**:
  Generally, for pre-analytical and analytical processing, use places decontaminated from DNA. We recommend to perform handling steps under UV-irradiated workstations. UV irradiation must be done before working according to the recommendations of the manufacturer. Routinely treat the surfaces of the working places with a commercial DNA decontamination reagent which is compatible with sterile protective gloves. Make sure that the material to be decontaminated is resistant to such treatment. Do not transfer supplies (e.g., pipettes, microcentrifuges, vortexer) and disposable material as specified by the handlings below from one working place to another. Each working place should be equipped with refrigerators (+4 to +12°C) and freezers (-15 to -25°C) for storage of the reagents of the kit. For transport of vials with mastermixes from one place to another, cooling rack should be used and a separate decontaminated with a decontamination reagent, e.g., DNA Exitus® after each transport.

- **Infectious material and cross-contamination**:
  Handle potentially infectious material with great care under a Class II biological safety cabinet in order to protect yourself from infection and avoid cross-contamination of samples and carry-over contamination of extraction buffers and reagents.

  Wear sterile protective gloves and sterile disposable sleeve covers at any handling step, including handling of infectious material, sample pre-treatment and PCR analysis. Frequently change protective gloves during handling. Use protective goggles, and disposable lab coats and overshoes and change when moving from one lab to another (below).

  Take care to maintain a DNA-free environment during opening the vials and bottles and handling the mastermixes. Close vials and bottles immediately after the removal of fluid.

  All DNA extraction buffers and reagents (DNA Isolation unit, kits 1 and 2) are assembled in 4 sample extraction units to minimise the risk of contamination of buffers and reagents during working with samples. Opened vials can be used within 4 days when stored under the conditions specified.

  Use only DNA-free pipette tips, vials and consumables recommended (page 9f.).

  Generally, run PCR negative and positive controls as well as internal controls with each series of assays to check for DNA contamination by handling during the preparation of mastermixes and the correct performance of the assays, respectively.

Part 1: Pre-Analytics

DNA Isolation

Use the following kits:

- ***Kit 1*** *(store at +18 to +25°C)*
  - *- Buffers (packages A, D and E)*
  - *- ST - Sample tubes (package B)*
  - *- SC - Spin columns (package C)*
  - *- CT - Collection tubes (package C)*
  - *- ET - Elution tubes (package C)*
- ***Kit 2*** *(store at -15 to -25°C)*
  - *- Enzymes and Reagents*
- ***Internal Extraction Control Unit,***
  in Kit 2 (store at -15 to -25°C)
  - *- Control DNA*

Part 1 - Pre-Analytics - DNA Isolation

Sample Collection

Because of the universal nature and the extreme sensitivity of detection of the assay, special care has to be taken for sample collection to avoid contamination by skin and environmental microorganisms. Transfer the samples to the laboratory for immediate processing (pages 22 to 44). Alternatively, store the samples in a refrigerator (+4 to +12°C). The stored sample must be analysed within 2 days after sample collection. For longer storage conditions, see the appropriate section (page 24, step 5) of the DNA isolation protocol.

Isolation of Pathogen DNA

Work in a place, which is ideally in a lab separated from places where mastermixes are handled and amplification is performed. Calibrate the procedure by spiking negative samples (buffer *SU*) with dilutions of full-grown cultures of pathogens or by using BioBall® MultiShot 550 KBE ('positive sample control', page 15). Please call Molzym for more information about positive sample controlling.

! For equipment, consumables and reagents to be supplied by the user see pages 8 to 9.

! To minimise cross-contamination, this unit is assembled in vials containing buffers or reagents for 4 sample extractions. Once removed for usage and opened, store buffers in a DNA-free environment at room temperature (+18 to +25°C) in the dark for up to 4 days. To avoid carry-over contamination, close caps of bottles/vials immediately after removal of solution. The vials of buffer *SU* are only intended for one sample each and must be discarded after use (incl. residual buffer).

! Take care that the enzymes and reagents of Kit 2 (*Enzyme K*, *MolDNase B, BugLysis, ß-mercaptoethanol* and *Proteinase K* solutions) are placed in a cooling rack adjusted to -15 to -25°C. After use, close caps of vials and replace vials to the freezer (-15 to -25°C).

Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.

! Before starting the protocol thaw the *Control DNA* (1 vial per 4 samples; Internal Extraction Control Unit, Kit 2) at room temperature (+18 to +25°C) (needed for step 8, page 24).

! Adjust the thermomixer to 37°C (needed for step 7, page 24).
Place vials of buffer *ES* (1 vial per 4 sample) into the thermomixer (needed for step 17, page 25). For tissue samples: Heat the thermomixer to 56°C for the pre-treatment (section 1B, iii. Tissue samples, page 23).

! Use only fresh samples. For blood collection, Molzym has evaluated K-EDTA and citrate S-Monovette® (Sarstedt, Germany) for the use with ***SepsiTest™-UMD***. After collection, the sample should be transported to the laboratory and processed immediately. If this is not possible, the sample should be placed in a refrigerator (+4 to +12°C), where it can be stored for 2 days at maximum. ***Do not freeze samples*** to avoid potential loss of pathogen DNA due to cell disruption as a result of freezing and thawing. If freezing of samples is desired, use Molzym's ***UMD-Tubes*** (order no. Z-801-020) which stabilise fluid samples. Thaw samples to room temperature (+18 to +25°C) for extraction. You can also interrupt the following procedure of pathogen DNA isolation at a certain step (step 5, page 24).

! Per sample, mark a *Spin column* (*SC*), two *Collection tubes* (*CT*, 2.0 ml) and one *Elution tube* (*ET*, 1.5 ml) of Kit 1C with a permanent marker for identification of the sample.

! Leave items used for the workflow (e.g., pipettes, racks, pipette boxes) in the workstation and have them exposed to the UV irradiation for decontamination before starting. In case of contamination of pipettes and other items or spilling the surface of the workstation by sample material, decontaminate as advised (Safety Information, page 10). Arrange all items according to your personal customs.

Protocols

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat and protective goggles when handling infectious material. Work in a Class II biological safety cabinet irradiated with UV before starting according to the instructions of the manufacturer. Follow the instructions of the manufacturer for maintenance of the workstation. Use fresh pipette tips with each pipetting step.

1A) How to Start

- **Kit 1** (+18 to +25°C) and **Internal Extraction Control Unit** (Kit 2, -15 to -25°C).

  Kit 1 contains buffers and consumables (single packages A to E) for the extraction and isolation of DNA from patient samples.

  Internal Extraction Control Unit contains the *Control DNA* (in bags, -15 to -25°C).

  Open the packages and vials only in the Class II biological safety cabinet. Thaw the Control DNA at room temperature (+18 to +25°C; needed for step 8, page 24).

  Shortly centrifuge the buffer vials (Kit 1).

  Arrange all the vials for the needed sample numbers (Kit 1 and Internal Extraction Control Unit in Kit 2) in a separate rack according to the sequence of steps as below:

  SU (for fluid samples and swabs) or PKB – TSB (for tissue)

  CM – DB1 – RS – RL – Control DNA– RP – CS – AB – WB – WS – ES →

  Excess vials of buffers and consumables of the opened packages can be stored at room temperature (+18 to +25°C) in a dark, DNA-free place for 4 days.

  The opened buffer *TSB* can be stored at room temperature (+18 to +25°C) in a dark, DNA-free place to the expiry date of the Kit.

  The opened buffer *SU* must be discarded. Each vial is intended for single use per sample.

- **Kit 2** contains the enzymes and reagents (in white boxes, -15 to -25°C).

  Take care that *Enzyme K*, *MolDNase B*, *BugLysis*, *β-mercaptoethanol* and *Proteinase K* solutions are kept at -15 to -25°C throughout. Replace enzymes and the reagent to the freezer (-15 to -25°C) immediately after handling.

  Caution: Make sure that the enzymes are not frozen when pipetting. Before use, vortex the enzymes and shortly centrifuge the vials to clear the lid.

1B) Arrangements and Pre-Treatment of Samples

i) Body fluids

(ascites, BAL, blood, CSF, plasma, pleural fluid, pus, synovial fluids)

- Fluid specimens are sampled under conditions avoiding contamination (page 19) and transported to the laboratory.
- For the isolation of blood samples use only K-EDTA- or citrate-stabilised whole blood.
- Pipette 1 ml fresh fluid sample from the sample container into a *Sample tube* (*ST tube*; Kit 1). In case of sample volumes less than 1 ml, pipette the fluid into the *ST tube* and fill up to 1 ml with buffer *SU* (Kit 1) (use the measure line of the tube). Continue with step 1 of the DNA Isolation Protocol (section 1C; page 24).

ii) Swabs (mouth, nasopharynx, wounds, bones)

- Pipette 1 ml of buffer *SU* (Kit 1) into a *Sample tube* (*ST tube;* Kit 1). If there is fluid in the swab vial, pipette 1 ml thereof into a *ST tube* instead of buffer *SU*. In case of less sample volume available, fill up to 1 ml by pipetting buffer *SU* to the sample in the *ST tube* (use the measure line of the tube).
- Remove the swab from the swab vial and transfer to the *ST tube*.
- Wash the swab by swirling in the fluid and pressing to the wall of the *ST tube* several times. Thereafter discard the swab. Continue with step 1 of the DNA Isolation Protocol (section 1C; page 24).

iii) Tissue samples (abscesses, biopsies, heart valves, prostheses)

- Tissue specimens are sampled under conditions avoiding contamination (page 19) and transported to the laboratory.
- Pipette 180 µl of buffer *PKB* (Kit 1) into a *Sample tube* (*ST tube*, Kit 1).
- Transfer the specimen to a sterile support, e.g., a Petri dish, by using sterile forceps. For preparation of the tissue specimen, the area should measure at maximum approx. 0.5x 0.5 x 0.5 cm. Cut the specimen into small pieces by using a sterile scalpel or sterile preparation scissors. Thereafter, transfer the cut specimen to the *ST tube* filled with buffer *PKB*. Add 20 µl of *Enzyme K* (Kit 2) to the specimen.
- Vortex the *ST tube* at full speed for 15s and incubate in the thermomixer at 56°C and 1,000 rpm for 10 min.

  After the incubation, adjust the thermomixer to 37°C.

  Comment: The tissue is partially digested and may decay. Potentially present bacteria and fungi are released from biofilms.
- Fill up to 1 ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube) and go to step 1 of the DNA Isolation Protocol (section 1C; page 24).

1C) DNA Isolation Protocol

1. **Pipette 250 µl buffer *CM* to the *ST tube* containing the sample (section 1B, page 23). Vortex at full speed for 15 s to mix properly. Let stand on the bench at room temperature (+18 to +25°C) for 5 min.**

   Buffer *CM* is a chaotropic buffer that lyses the human cells.

   Caution: Buffer *CM* is an irritant. Avoid contact with skin and eyes.

2. **Briefly centrifuge to clear the lid. Pipette 250 µl buffer *DB1* to the *ST tube*. Thereafter pipette 10 µl *MolDNase B* to the lysate in the *ST tube*. Immediately vortex for 15 s properly and let stand for 15min at room temperature (+18 to +25°C). Replace the vial with *MolDNase B* to -15 to -25°C for further storage.**

   During this step the nucleic acids released from human cells are degraded.

3. **Centrifuge the *ST tube* in a bench top microcentrifuge at ≥12,000x*g* for 10 min. Thereafter, carefully remove the supernatant by pipetting and discard.**

   Human cell debris and potentially present pathogen cells are sedimented.

4. **Pipette 1 ml buffer *RS* to the sediment and resuspend by pipetting.**
   Depending on the specimen, the sediment may contain residues of tissue and may be rigid due to debris. Resuspension may take some time.

5. **Centrifuge the *ST tube* in a bench top microcentrifuge (≥12,000x*g*) for 5 min. Carefully remove the supernatant by pipetting and discard.**

   This washing removes residual *MolDNase B* activity, chaotropic salts and part of the PCR inhibitors.

At this point the procedure can be interrupted by freezing the sample (-15 to -25°C). For further processing, thaw the sample at room temperature (+18 to +25°C) and proceed with step 6.

6. **Pipette 80 µl buffer *RL* to the *ST tube*. Resuspend the sediment by vigorous vortexing. Briefly centrifuge to clear the lid.**

   Pipette 20 µl *BugLysis* directly into the extract in the *ST tube* and then add 1.4 µl *ß-mercaptoethanol*. Vortex the tube for 15 s and incubate in a thermomixer at 37°C and 1,000 rpm for 30 min. Store non used *BugLysis in the* vial at -15 to -25°C for up to 4 days.

   The cell walls of potentially present bacteria and fungi are degraded.

   Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.

   After the incubation, adjust the thermomixer to 56°C.

7. **Note:** Preparation in this step, do not work in the sample tube itself.

   Pipette 10 µl of the *Control DNA* into a vial of buffer *RP* (4 samples) and vortex the tube for 15 s. Briefly centrifuge the vial to clear the lid. Continue to *ST* tube.

8. **Briefly centrifuge the *ST tube*. Add 150 µl buffer *RP* incl. the *Control DNA* to the *ST tube*. Then pipette 20 µl *Proteinase K* directly to the extract in the *ST tube*. Vortex the *ST tube* for 15 s and incubate at 56°C and 1,000 rpm for 10 min.**

   Store not used *Proteinase K* in the vial at -15 to -25°C and opened *RP* vials incl. *Control DNA* for up to 4 days (+18 to +25°C).

   After the incubation, adjust the thermomixer to 70°C (make sure that the vials of buffer *ES* are placed in the thermomixer (ES is needed at step 15).

9. **Briefly centrifuge the *ST tube* and pipette 250 µl buffer *CS* into it. Vortex tube at full speed for 15 s.**

   Cells are lysed and protein is denatured.

10. **Briefly centrifuge the *ST tube*. Pipette 250 µl binding buffer *AB* to the *ST tube*. Vortex the tube at full speed for 15 s.**

11. **Briefly centrifuge the *ST tube* and transfer the lysate to a *Spin column* (*SC*; Kit 1, package C) pre-assembled in a 2.0 ml *Collection tube* (*CT*) by pipetting.** **Tissue samples:** **Do not transfer undigested material** (pulse centrifuge to sediment and pipette supernatant. You may want to use a smaller pipette tip to avoid transferring particles).
    Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).

    At this point nucleic acids bind to the matrix.

12. **Remove the closed *Spin column* from the centrifuge. Open the lid, remove the *Spin column* and insert it into a new 2.0 ml *Collection tube* (*CT*; Kit 1, package C). Discard the *Collection tube* with flow-through. Pipette 400 µl buffer *WB* to the *Spin column*. Close the lid and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).**

13. **Remove the closed *Spin column* from the centrifuge. Open the lid, remove the *Spin column and insert it into a new 2.0 ml Collection tube /CT; Kit 1, package C).* Discard the *Collection tube* with flow-through. Pipette 400 µl of buffer *WS* to the *Spin column*. Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 3 min.**

    This step removes salts and dries the column matrix.

14. **Carefully remove the closed *Spin column* from the centrifuge. Avoid splashing of the flow-through to the *Spin column*. Remove the *Spin column* from the *Collection tube* and place into a 1.5 ml *Elution tube* (*ET*; Kit 1, package C). Discard the *Collection tube* containing the flow-through.**

15. **Place 100 µl buffer ES (vials in the thermomixer already preheated to 70°C), in the centre of the column, close lid and incubate for 1 min at room temperature (+18 to +25°C). Thereafter, centrifuge at ≥12,000xg for 1 min to elute the DNA. Finally, remove the *Spin column* from the *Elution tube* and close the lid. Discard the *Spin column*.**

16. **Store the *Elution tube* containing the eluate at +4 to +12°C if analysed at the same day or freeze at -15 to -25°C until further use (part 2, pages 26 to 35).**

    Avoid frequent freeze-thaw cycles, because this may result in a decay of the eluted DNA (in particular at low DNA concentrations).

Part 2: Analytics

PCR Detection and Sequence Identification

Protocols for PCR, Gel Electrophoretic Analysis & Sequencing

Addendum:
Real-Time PCR Protocols

Use the following kits of the PCR Detection & Identification Unit:

- ***Kit 3*** *(store at -15 to -25°C)*
  – PCR Reagents
- ***Kit 4*** *(store at -15 to -25°C)*
  – PCR Controls & Detection Reagents
- ***Consumables PCR Detection & Identification (Kit 1)*** *(store at +18 to +25°C)**:***
  – MT - Mastermix tubes, 1.5 ml

Part 2 – Analytics

Description of the Assays

With this unit, PCR assays are supplied for the testing of the presence of bacterial and fungal DNA in clinical specimens. The 'Mastermix Assay Bacteria' (*MA Bac*, Kit 3) and 'Mastermix Assay Yeasts' (*MA Yeasts*, Kit 3) are based on primers that bind to conserved regions of the 16S (V3/V4 region) and 18S (V8/V9 region) rRNA genes of bacteria and fungi, respectively. The tests comprise a two-step procedure including the use of **i)** the mastermixes *MA Bac* and *MA Yeasts* (Kit 3) for the PCR amplification of sequences using extracted DNA (pages 20 to 25) and **ii)** agarose gel electrophoresis for the detection of amplified DNA, using components supplied with Kit 4. Alternatively, protocols for the Real-Time PCR detection are supplied with the Addendum (page 36 to 44).
MA Bac and *MA Yeasts* are 2.5x concentrated solutions, the final volume of the reaction mixture being 25 µl. This PCR Detection unit contains all components necessary for PCR runs.
The 'Mastermix Assay Control' (*MA Control*, Kit 3) is a control for the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. The assay tests the presence of the *Control DNA* (Internal Extraction Control Unit), which is added in the extraction process and is included in the eluate of the samples. The correct function of the extraction process and the absence of PCR inhibitors potentially co-eluted with the target pathogen DNA are monitored by *MA Control*.

Protocols for amplification are supplied for the following instruments:

- Thermal Cycler (protocol, page 32):
  - Mastercycler® Eppendorf
- Real-Time PCR instruments (addendum pages 37 to 40):
  - LightCycler® 1.5, 2.0, 96, 480 and Nano, Roche
  - DNA Engine Opticon®, CFX96™, BioRad
  - Mx3000P®, Mx3005P®, Stratagene
  - ABI 7500 Fast®, Life Technologies
  - Rotor-Gene®, Qiagen
  - peqStar 96Q, peqlab

If using other instruments, make sure that the Assays *MA Bac*, *MA Yeasts* and *MA Control* perform correctly with the cycler. For *MA Bac* and *MA Yeasts*, perform PCR reactions using PCR DNA Standard P1 and P2 which both should result in an amplification product. For *MA Control* a negative sample control (*SU* buffer, Kit 1) is extracted and tested in the assay. See PCR Detection, section 2A to 2E for the procedure (pages 28 to 34).

Packaging, Storage and Handling

Store all vials in this unit (Kits 3 and 4) at -15 to -25°C upon receipt.

The purification and packaging of the mastermixes supplied in this PCR detection unit are performed under standard precautions for the avoidance of air-borne and handling-based DNA contaminations. The mastermixes are supplied as a 2.5x concentrated solution in DNA-free screw cap vials (Kit 3). For usage, the mastermixes are thawed at room temperature (+18 to +25°C). After use, the mastermixes can be stored in the refrigerator (+4 to +12°C) for further use at the same day, but should be replaced to -15 to -25°C for longer storage.

MolTaq 16S/18S has to be kept at -15 to -25°C throughout handling (cooling rack). Replace *MolTaq 16S/18S* to the freezer (-15 to -25°C) after handling. **Caution:** Make sure that the *MolTaq 16S/18S* is not frozen when pipetting. Before use, vortex the *MolTaq 16S/18S* and shortly centrifuge the vial to clear the lid.

It is important to note that the DNA staining solution (*DS*, Kit 3) and DNA size marker (*SM*, Kit 4A) are sensitive to light and should be stored in the dark during handling and storage. **Once thawed, do not freeze again** and store at +4 to +12°C for further use.

Store the *Gel loading solution* (*LS*, Kit 4A) after first handling at +4 to +12°C.
Store *DNA dilution buffer* and *DNA Standard P1* (Kit 4B) at +4 to +12°C after thawing.

Take care to maintain a DNA-free environment during opening the vials and handling the mastermixes by working under a UV-decontaminated workstation. Use only certified microbial DNA-free pipette tips and PCR consumables recommended for running the assays (pages 8 to 9).

Quality Control and Specifications

Negative PCR controls using supplied DNA-free water instead of eluate are used for routine detection of contamination by microbial DNA in the purified final mastermixes (*MA Bac* and *MA Yeasts*). Guarantee is given for the absence of signals in negative controls at a rate of ≤5 %, provided the avoidance of contamination by handling errors.

Positive PCR controls should always be run and contain a high (P1) and low (P2) concentrated target DNA amount per assay. *DNA Standard P1* is supplied with Kit 4B and serves as a run control of the PCR reaction. P2 is diluted from P1 and indicates the sensitivity of the assays, *MA Bac* and *MA Yeasts*. The Standard DNA is a mixture of known amounts of genomic DNA from *Bacillus subtilis* and *Saccharomyces cerevisiae*.

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles, a bouffant cover and disposable overshoes during the setup of mastermixes. Work in a PCR workstation irradiated with UV before starting according to the instruction manual of the manufacturer. Follow the instructions of the manufacturer for maintenance of the PCR workstation. Do not work under UV irradiation.

PCR Detection and Identification of Pathogens by Sequencing

PCR Detection

Assay *MA Bac* and *MA Yeasts*:
With each mastermix an extra volume is supplied to run PCR controls. Negative PCR controls should always be performed, at least with 10 % of the sample runs, to test for potential cross-contamination or other carryovers resulting from handling or air-borne errors. Also, with each set of sample runs positive PCR controls (page 16) must be included to control the performance of the assay. Follow the instructions for the performance of positive PCR controls (pages 29 to 32).

Assay *MA Control*:
The Assay *MA Control* (Internal Extraction Control; Kit 3) is a test to check the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. *MA Control* has to be performed with each sample testing (pages 29 to 32).

Validity of results:
Only if the negative PCR controls (*MA Bac*, *MA Yeasts* and *MA Control*) lack a PCR signal, the positive PCR controls (P1 and P2) and the positive Internal Extraction Control result in a band of specific size in the gel electrophoresis analysis, the results of the sample test can be considered valid results.

Exceptional cases:

If the sample lacks a PCR signal, the Internal Extraction Control is positive and the negative PCR controls *MA Bac* or *MA Yeasts* show a positive result, which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit. In this case the negative result of the sample is valid.

In case of a specific signal in the sample test and the Internal Extraction Control lacks a signal, the result of the sample is valid. In this case, all PCR controls must perform as expected.

2A) How to Start

! For equipment, consumables and reagents to be supplied by the user see pages 8 to 9.

Avoidance of DNA Contamination

! To avoid contamination it is important that the setup of *MA Bac* and *MA Yeasts* is performed in a lab separated from DNA extraction and PCR amplifications.

! For each pipetting use fresh tips.

! Take care that all handling is performed in a DNA-free environment.

! To avoid contamination, close caps immediately after removal of solution.

For more details, see page 19.

Storage of the PCR Reagents after Handling

! After use, keep the 2.5x mastermixes and H_2O in a refrigerator (+4 to +12°C) if reused at the same day or store at -15 to -25°C for longer periods

! Replace *MolTaq 16S/18S* in a cooling rack (-15 to -25°C). Always keep and store *MolTaq 16S/18S* at -15 to -25°C. **Do not interrupt the cooling of *MolTaq 16S/18S*.**

! After first use, store DNA staining solution (*DS*) in the dark at +4 to +12°C. **Do not re-freeze**.

! After the first use, store *DNA dilution buffer* and *DNA Standard P1* at +4 to +12°C.

! Diluted DNA solutions (P2) tend to be unstable. Always prepare P2 freshly for each series of PCRs (step 8, page 32).

For more information about the storage and stability of the PCR reagents see Tab. 1, page 6.

Places where Handlings are performed

Symbols and explanation of the PCR working places:

DNA-free

Work under a PCR UV workstation. Use components of **Kit 3** and **consumables** (*MT*, Mastermix tubes for Kit 3) in **Kit 1**.
For the preparation of mastermixes *MA Bac, MA Yeasts* and *MA Control*.

DNA handling

Work under a UV Class II biological safety cabinet where samples are prepared. Use components of **Kit 4B.** For the preparation of:

- Sample loading into the assays
- Positive PCR controls P1 and P2

PCR Assays

Per assay (*MA Bac, MA Yeasts* and *MA Control*), the following PCR reactions have to be run:

DNA-free

MA Bac
- 1 reaction per sample
- 2 reactions for the positive controls (P1, P2)
- 1 reaction for negative control (NC Bac)

MA Yeasts
- 1 reaction per sample
- 2 reactions for the positive controls (P1, P2)
- 1 reaction for negative control (NC Yeasts)

MA Control (*Internal Extraction Control*, IEC)
- 1 reaction per sample
- 1 reaction for negative control (NC IEC)

Thaw the following vials at room temperature (+18 to +25°C):

DNA-free

Kit 3:
- H_2O
- 2.5x *MA Bac*
- 2.5x *MA Yeasts*
- 2.5x *MA Control*
- *DS;* keep dark

DNA

Kit 4B:
- *DNA Standard P1*
- *DNA dilution buffer* (for P1)

Before starting with the preparation of PCR-ready mastermixes (section 2B, page 31):

Vortex thawed PCR reagent vials (Kit 3) for a few seconds to mix and briefly centrifuge to clear the lid.

2B) Setup of the Assays

Keep all PCR tubes filled with PCR-ready mastermix and *MolTaq 16S/18S* chilled in the cooling racks, until placing in the PCR cycler. Cooling of the PCR tubes is important to minimize the generation of primer dimers.

DNA-free

Preparation of PCR-ready mastermixes *MA Bac*, *MA Yeasts* and *MA Control*

1. **Arrange the PCR tubes for *MA Bac*, *MA Yeasts* and *MA Control* in a PCR cooling rack (-15 to -25°C) and mark (PCR Assaying, page 30).**
2. **Place *MolTaq 16S/18S* (Kit 3) in the cooling rack (-15 to -25°C).**
3. **Use a *MT* (*Mastermix tube* 1.5 ml for Kit 3; in Kit 1) for *MA Bac*, another *MT* tube for *MA Yeasts* and a third *MT* tube for *MA Control*.**
   Place the *MT* tubes in a cooling rack. Pipette the supplied components of Kit 3 (Tab. 5) into each *MT* tube. Vortex the tubes to mix and briefly centrifuge.
4. **Pipette 20 µl of the PCR-ready mastermix *MA Bac* into each PCR tube (dedicated for samples, P1, P2 and NC, respectively). Repeat the procedure with mastermix *MA Yeasts*. Pipette 20 µl of the PCR-ready mastermix *MA Control* into each PCR tube (dedicated for samples and NC).**
5. **Add 5 µl *H_2O* (DNA-free water; Kit 3) into the NC PCR tubes. Close all PCR tubes with the caps.**
6. **Place the PCR tubes in another cooling rack designated for transport to the UV Class II biological safety cabinet.**

DNA handling

Sample loading for assays *MA Bac, MA Yeasts* and *MA Control*

7. **Pipette 5 µl of each sample eluate into the PCR tubes containing *MA Bac*, *MA Yeasts* and *MA Control*, respectively. Close the PCR tubes.**

Positive PCR controls *P1* and *P2*

8. **Vortex the *P1* vial and pulse centrifuge. Pipette 998 µl of *DNA dilution buffer* into a 1.5 ml sterile polypropylene tube (not supplied). Add 2 µl *DNA Standard P1* and vortex to mix (*P2*). Briefly centrifuge.**
9. **Pipette 5 µl of positive PCR control *P2* into a PCR tube containing *MA Bac* and *MA Yeasts*, respectively. Repeat with *P1*. Close PCR tubes with caps.**
10. **Continue with section 2C PCR Thermocycling page 32.**

Tab. 5: Preparation of PCR-ready mastermixes (Kit 3). Volumes in µl.

reactions	*MA Bac*, *MA Yeasts* or *MA Control*	*H_2O*	*DS*	*MolTaq 16S/18S*
1	10.0	7.5	2.5	0.8
2	20.0	15.0	5.0	1.6
3	30.0	22.5	7.5	2.4
4	40.0	30.0	10.0	3.2
5	50.0	37.5	12.5	4.0
6	60.0	45.0	15.0	4.8
7	70.0	52.5	17.5	5.6
8	80.0	60.0	20.0	6.4
9	90.0	67.5	22.5	7.2
10	100.0	75.0	25.0	8.0

2C) PCR Thermocycling

Transport all chilled PCR tubes (strips or plates) prepared as above to a place where PCR runs are performed. Programme the Mastercycler (Eppendorf) as described (Tab. 6). After the PCR run go to section 2D for the detection of amplicons.

Tab. 6: PCR programme for Mastercycler (Eppendorf)

Method	Cycles	Target temperature [°C]	Incubation time [hh:mm:ss]
Initial denaturation	1	95	00:01:00
Cycling	40	95	00:00:05
		55	00:00:05
		72	00:00:25
Cooling	1	10	00:00:10

2D) Detection by Agarose Gel Electrophoresis

After thermocycling, transport the PCR tubes to a place where DNA is handled. Use components of Kit 4A.

! For equipment, consumables and reagents to be supplied by the user (page 9).

! The DNA staining solution (*DS*), which is present in the mastermixes during PCR amplification, is used for gel electrophoretic visualisation of the amplicon DNA. Make sure that the tubes are kept in the dark until gel electrophoresis. Thaw the DNA size marker (*SM*). The DNA size marker should be kept at +4 to +12°C in the dark for further storage (**do not freeze again**).

Protocol:

Prepare a gel (2 %) following the instructions of the manufacturer or prepare a 2 % (w/v) agarose gel in 1x TAE buffer. Place the gel in a tray, transfer into the chamber and fill with freshly prepared 1x TAE running buffer as instructed by the manufacturer (the gel should be covered with approx. 1cm buffer).

- Per PCR reaction, pipette 8 µl of the PCR product into a sterile 1.5 ml polypropylene tube (not supplied) or in a well of a 96 well plate (not supplied) and mix with 2 µl of the gel loading solution (*LS*). Mix by pipetting in and out for several times.
- Pipette the mixture (10 µl) into an indentation of the gel. Repeat the procedure with the other PCR product, including samples, Internal Extraction Controls, positive PCR controls (P1 and P2), negative PCR controls and, at the end pipette 5 µl of supplied *SM* (DNA size marker; Kit 4A).
- Close the electrophoresis chamber with the cover and run the gel at 10 V/cm interelectrode distance in the dark.
- Leave the gel running until the blue dye has moved about 2/3 of the way through the gel. At the conditions described this takes about 30 to 45 min.
- Remove the gel, place under a UV lamp or on a transilluminator (260 to 310 nm wavelength) and photograph/document. Compare appearing bands of samples with the DNA size marker and positive PCR controls P1 and P2. For an example, see Fig. 3 and Fig. 4, pages 33 to 34.
  Note: If all bands showed a too weak fluorescence signal, the gel can be re-coloured in e.g. an ethidium bromide staining bath, if necessary.
- Make sure that bands appear within the samples in the assay *MA Control* (Internal Extraction Control). This is important in cases of negative samples. Bands in this assay indicate the absence of PCR inhibitors in the eluates and the correct performance of the extraction process.

2E) Guidance to the Interpretation of PCR Results

A typical image of the results of the analysis of samples is given in Fig. 3. In this case, samples were collected from six patients and processed as described in the previous sections.
The controls were as expected, i.e., positive PCR controls P1 and P2 showed bands at the correct position (approx. 450bp, see 'bacterial DNA' in Fig. 3).
The negative PCR controls (NC) did not show a signal, so DNA contamination can be excluded.
The Internal Extraction Controls C1 to C5 of the samples 1 through 5 (Fig. 3) showed clearly visible bands (approx. 200bp), demonstrating the function of the extraction process. The results from the Internal Extraction Controls, positive PCR controls and negative PCR controls indicate that the assay performed correctly.
Five of the samples showed bands (samples 1 to 3, 5, 6) in the assay *MA Bac* at the expected gel position (Fig. 3) and thus were positive for bacterial DNA. Sample 4 (Fig. 3) was negative (only showing weak bands at approx. 320bp, indicating traces of amplified human DNA; 'human DNA', Fig. 3).

Banding of amplification products from Assay *MA Yeasts* (fungi) appear at a position of approx. 310bp (Fig. 4, page 34).

Exceptional cases:
In the case of the specific bacterial band in the sample test (6, Fig. 3) but lacking signal in the Internal Extraction Control (C6), the positive PCR sample result is valid.

All positive PCR samples have to be identified by sequencing.

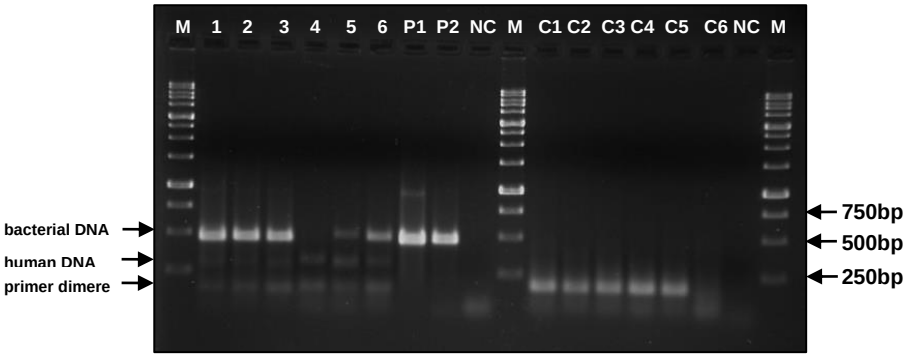


Fig. 3: PCR analysis of eluates from 6 patient samples (1 to 6) using Mastermix Assay Bacteria (*MA Bac*). *P1, P2*: Positive PCR controls; NC: Negative PCR controls; C1 through C6: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples 1 to 5 (6: negative) (banding at approx. 200bp); M: DNA size marker (*SM*). The weak unspecific signals (left side of the figure; arrow 'human DNA', approx. 320bp) below the specific signals from samples (arrow 'bacterial DNA', approx. 450bp) are the result of the amplification of traces of human DNA, well separated from the specific bands.

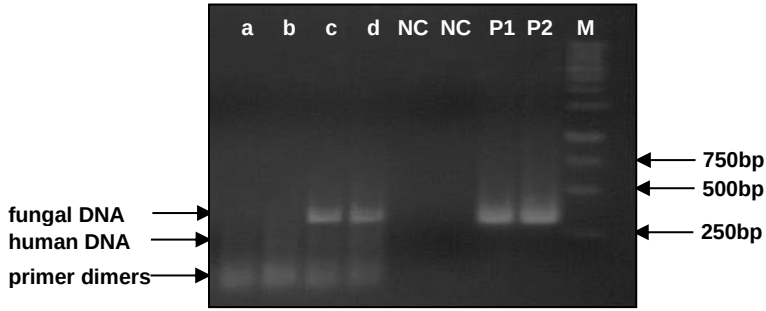


Fig. 4: PCR analysis of eluates from four patient samples (a to d) using Mastermix Assay Yeasts (*MA Yeasts*, fungi). P1, P2: Positive PCR controls; NC: Negative PCR control; M: DNA size marker (SM). Signals at the position 'fungal DNA' (310bp) indicate that samples of patients c and d contain fungal DNA (sequencing result: *Candida albicans*). The weak signals at approximately 250bp (arrow 'human DNA') are the results of the amplification of traces of human DNA.

Identification of Pathogens by Sequencing of Amplicons

All positive PCR samples have to be identified by sequencing.
Sequencing of amplicons together with BLAST online homology search is used for the identification of pathogens detected by ***SepsiTest™-UMD***. Sequence analysis has been validated with ***SepsiTest™-UMD***. Online BLAST tools are available, e.g., NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The free online tool, SepsiTest™-BLAST, is a user friendly way of identification of pathogens relying on quality-controlled reference data sets of more than 7,000 sequences from cultured bacteria and more than 340 sequences from cultured *Candida* spp, *Cryptococcus* spp. and *Aspergillus* spp. (http://www.sepsitest-blast.com). For further investigations see section 2H, page 35.

2F) Purification of Amplicons

For sequencing, amplicons need to be purified. Qiagen's QIAquick® PCR Purification Kit (cat. no. 28104) has been validated with ***SepsiTest™-UMD***. For this purpose, use the aliquot remaining after analysis of the PCR product (approximately **17 µl**; 25 µl, if using Real-Time PCR; addendum pages 36 to 44) and follow the instructions of the manufacturer of the kit. Elute the purified amplicon from the column (QIAquick®) with **30 µl** sterile deionised water.
Continue with the sequencing procedure (section 2G, page 35).

2G) Sequencing

Apply the purified amplicon DNA to a sequencing reaction as advised by the manufacturer of the sequencing system. ***SepsiTest™-UMD*** has been validated using Applied Biosystems DNA Analyzer ABI 3730XL® and ABI Prism310® apparatuses together with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For sequencing, use the sequencing primers (10 µM each) supplied with Kit 4. For sequencing of amplicons from Assay *MA Bac* (bacteria) use *SeqGP16* and *SeqGN16* in separate reactions, and from Assay *MA Yeasts* (fungi) use *SeqYeast18*. *SeqGP16* and *SeqGN16* are primers binding to regions within the amplicon specific for Gram-positive and Gram-negative bacteria, respectively. *SeqGN16* targets mainly Gram-negative bacteria, including few gram-positive bacteria. The excluding gram-negative species will detect by *SeqGP16*. *SeqGP16* targets mainly Gram-positive bacteria, including few gram-negative species. The excluding species will be detect by *SeqGN16*. The exceptions of the sequencing primers see in table on at the following link:
https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf
As an example, the following protocol for QIAquick®-purified amplicons using the ABI Prism310® may give satisfying results. Use 2 µl of purified DNA for cycle sequencing. Add 4 µl Big-Dye® Reaction mix (containing polymerase und nucleotides), 0.5 µl sequencing primer *SeqGP16*, *SeqGN16* or *SeqYeast18* (10 pmol/µl) and PCR-grade water to fill up to a final volume of 20 µl. Incubate in a PCR machine under the following conditions: Initial denaturation at 95°C for 1 min; 26 cycles at 95°C for 30 s, 55°C for 30 s and 60°C for 4 min. To remove dye terminator molecules from sequencing samples use your internal validated process. Combine 5 µl of the eluate containing the products of the sequencing reaction with 20 µl formamide (or TSR reagent containing formamide) and incubate at 95°C for 4 min. Apply the reaction mix to the capillary of the ABI Prism310®.
Validate the performance of the used sequencing system. For this, analyse the purified amplicons of the positive PCR controls P1 and P2. Both controls should give readable results. Alternatively use an overnight sequencing service (e.g., Eurofins Genomics, Germany).

2H) SepsiTest™-BLAST Analysis for Strain Identification

Molzym has developed a free online service (www.sepsitest-blast.com) for the identification of bacteria and fungi based on small subunit rRNA genes. The identification relies on an algorithm for the comparison of input sequences with a reference sequence data library. SepsiTest™-BLAST is characterised by a pool of more than 7,000 quality-controlled complete sequences of the 16S and 18S rRNA genes of only cultured and denominated eubacteria, *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. The tool is very simple to use and results are obtained as an output of hits in the order of decreasing sequence identity scores.
An overview of all species covered by the SepsiTest™-BLAST database is given in an Excel file which can be downloaded in the FAQ section of the SepsiTest™-BLAST homepage (http://www.sepsitest-blast.com/en/faqs.html).
Please note: For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST http://www.ncbi.nlm.nih.gov/blast The result with the best score is valid.

Note: Sequence identities ≥97 to <99 % should be interpreted as on the genus level, ≥99 % as on the species level (Wellinghausen et al. 2009). Sequence identities below 97 % are rejected by SepsiTest™-BLAST. This may be the result of reading errors of the sequencing reaction. In such a case it is recommended to inspect the densitogram read-out for overlying sequences indicating the presence of more than one strain in the sample (Fig. 2, page 14). Overlying sequences can be resolved using a specialised tool, RipSeq® (Pathogenomix; http://www.ripseq.com).

Addendum: Real-Time PCR Protocols

In the following, protocols for Real-Time PCR are provided which are based on extensive evaluation to demonstrate their performance.

Please note that ***SepsiTest™-UMD*** does not provide a licence for the use of Real-Time PCR (see legal aspects, below). In the following, protocols are described for Roche LightCycler® 1.5, 2.0, 96, 480 and Nano Real-Time PCR machines, BioRad Opticon® DNA Engine and CFX96™ machines, ABI 7500 FAST, Stratagene Mx3000P® and Mx3005P® machines, Qiagen Rotor-Gene® and Peqlab peqStar 96Q. Other instruments may be validated for their use with this kit by the user. At the end, (pages 40 to 44) a guidance to the interpretation of possible results is given.

Patents/Disclaimer

Use of this product is limited to PCR as described in the previous sections (pages 26 to 34). Other applications, in particular Real-Time PCR, for which this product is described below, is covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application other than covered by patents of Molzym, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used. In particular, the patents for real-time PCR and the use of intercalating fluorescent dyes and probes, including their specific applications.

General Requirements

Please take notice of the general requirements for the performance of PCR (part 2, pages 28 to 32).

! Calibrate your Real-Time PCR machine using the Assays *MA Bac*, *MA Yeasts* and *MA Control* (Kit 3). For *MA Bac* and *MA Yeasts*, perform Real-Time PCR runs with supplied *Standard DNA P1* and *P2*. For *MA Control* a negative sample control (*SU* buffer, Kit 1) is extracted and tested in the assay.

The PCR run conditions are as according to the protocol described on pages 29 to 32. The specific thermocycling conditions are described on pages 37 to 39. For the preparation of mastermixes, follow the instructions (part 2, sections 2A to 2C, pages 29 to 32). Both positive PCR controls, P1 and P2 (*MA Bac* and *MA Yeasts*), and the sample eluate (*MA Control*) must show a target-specific peak (see page 40).

! For equipment, consumables and reagents to be supplied by the user see pages 8 to 9. In addition, the following items are needed to perform Real-Time PCR:

- 1x Real-Time PCR machine (above). Other instruments have to be validated for their use.
- PCR tubes e.g., glass capillaries (20 µl) for Lightcycler®1.5 (25 µl final volume per assay) or PCR strips (8x 0.2 ml) for other systems; e.g., Biozym Flat Optical 8-Cap Strip (order no. 712100)

! To avoid contamination, it is important that the setup of *MA Bac*, *MA Yeasts* and *MA Control* is performed in a lab separated from DNA extraction and PCR amplifications.

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles, a bouffant cover and disposable overshoes during the setup of mastermixes. Work in a PCR workstation irradiated with UV before starting according to the instruction manual of the manufacturer. Follow the instructions of the manufacturer for maintenance of the PCR workstation. Do not work under UV irradiation.

Real-Time PCR Thermocycling and Detection by Melting Curve Analysis

A melting curve analysis has always to be performed in order to discriminate possible primer dimer formation from true pathogen signals. See examples on pages 39 to 44. All positive Real-Time PCR sample results have to be identifying by sequencing. For sequencing of amplicons see part 2, section 2F to 2H, pages 34 to 35.

I) Roche LightCycler® 1.5 and 2.0 (25 µl final volume per assay)

Transport filled capillaries to a place where PCR runs are performed and programme the Real-Time PCR machine as described below. Set the appropriate channel to SYBR® Green 1 detection.

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.00	-	NONE
Cycling	40	Quantification	95	00:00:05	4.00	-	NONE
			55	00:00:05	4.00	-	NONE
			72	00:00:25	4.00	-	SINGLE
Melting	1	Melting Curve	95	00:00:00	20.00	-	NONE
			65	00:00:15	20.00	-	NONE
			95	00:00:00	0.05	-	CONT
Cooling	1	None	40	00:00:05	20.00	-	NONE

II) Roche LightCycler® 96

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.40	-	None
Cycling	40	Quantification	95	00:00:05	4.40	-	None
			55	00:00:10	2.20	-	None
			72	00:00:25	4.40	-	Single
Melting	1	Melting Curve	95	00:00:01	4.40	-	None
			70	00:00:01	2.20	-	None
			95	-	0.2	5	Continuous
Cooling	1	None	40	00:00:10	-	-	-

III) Roche LightCycler® 480

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.40	-	None
Cycling	40	Quantification	95	00:00:05	4.40	-	None
			55	00:00:10	2.20	-	None
			72	00:00:25	4.40	-	Single
Melting	1	Melting Curve	95	00:00:01	4.40	-	None
			70	00:00:01	2.20	-	None
			95	-	0.11	5	Continuous
Cooling	1	None	40	00:00:10	-	-	-

IV) Roche LightCycler® Nano

Set the appropriate channel to SYBR® Green I detection.

Method	Cycles	Programs	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	Hold	95	00:01:00	5.00	
Cycling	40	Quantification	95	00:00:05	2.00	
			55	00:00:05	2.00	
			72	00:00:25	2.00	✓ Acquire
Melting	1	Melting	60	00:00:20	4.00	
			95	00:00:20	0.1	
Cooling	1	Hold	40	00:00:05	5.00	

V) BioRad DNA Engine Opticon® and CFX96™

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	95	00:01:00		
Cycling	40	95	00:00:05		
		55	00:00:05		
		72	00:00:25		Reading point after 72°C step
Method	**Cycles**	**Melting Curve**			
Melting Curve	1	from 70°C to 95°C	Read every 0.2°C, hold for 1s between reads		

VI) ABI 7500 Fast®

Switch off the ROX reference.

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	95	00:01:00		
Cycling	40	95	00:00:05		
		55	00:00:10		
		72	00:00:25		on
Melting Curve	1	95	00:00:15		
		70	00:01:00		
		95		0.2	
		95	00:00:15		
Cooling	1	60	00:00:15	100 %	

VII) Stratagene Mx3000P® and Mx3005P®

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Amplification averaging point	Dissociation averaging points	Dissociation point separation
Initial denaturation	1	95	00:01:00			
Cycling	40	95	00:00:15			
		55	00:00:15			
		72 (reading point)	00:00:30			
Melting Curve	1	95	00:01:00			
		55	00:00:30			
		95		3	3	0.5°C

VIII) Qiagen Rotor-Gene®

To program a new run for melting curve analyses select: Three steps with Melt.

Amplification				
Method	**Cycles**	**Target Temperature [°C]**	**Incubation time [hh:mm:ss]**	**Acquisition Mode**
Hold	1	95	00:01:00	
Cycling	40	95	00:00:05	
		55	00:00:15	
		72	00:00:30	Acquiring to cycle A; Acquiring channel A

Melting				
Method	**Ramp Parameters**			**Acquire**
Melt	from	70	degrees	Melt A: on Green
	to	95	degrees	
	Rising by	0.2	degree(s) each step	
	Wait for	90	seconds of pre-melt conditioning on first step	
	Wait for	1	seconds for each step afterwards	
	Grain-Optimisation			
	☐ Optimise gain before melt on all tubes The gain giving the highest fluorescence less than will be selected			95

IX) Peqlab peqStar 96Q

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Step	Step Holding Sec.
Hold Stage	1	95	00:01:00	4		
PCR Stage	40	95	00:00:05	4		
		55	00:00:10	4		
		72 (Sampling)	00:00:25	4		
Melting Stage	1	95	00:00:01	4	0.1	00:01
		70	00:00:01	4		
		95 (Sampling)	00:00:01	4		
Infinite Stage	1	8	∞	4		

Guidance to the Interpretation of Real-Time PCR Results

This kit supplies assays for the amplification of the 16S and 18S rRNA genes of eubacteria and fungi, respectively. The advantage of this approach is that, in principle, all microorganisms are detected irrespective of the taxonomic status of the strain. The drawback, on the other side, of such a universal system is that the assays are prone to false positive results due to contamination by exogenous DNA introduced to the assays by aerosols or direct carryover between samples. Hence, the results of Real-Time PCR runs can lead to diverse appearances. In the following, besides true results, a selection of typical false results are presented and discussed (see examples pages 41 to 44).

Validity of results:

Only if the negative PCR controls (*MA Bac*, *MA Yeasts* and *MA Control*) lack a PCR signal, the positive PCR controls (P1 and P2) and the Internal Extraction Control result in a specific peak in the melting curve analysis, the results of the sample test can be considered valid results.

Exceptional cases:

If the sample lacks a PCR signal, the Internal Extraction Control is positive but the negative PCR controls *MA Bac* or *MA Yeasts* show a positive result, which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit. In this case the negative result of the sample is valid.

In case of a specific signal in the sample test and the Internal Extraction Control lacks a signal, the result of the sample is valid. Note that in this case all PCR controls must perform as expected.

Result Interpretation of Internal Extraction Control (*MA Control*):

The Assay *MA Control* (Internal Extraction Control; Kit 3) is a test to check the performance of the DNA extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. *MA Control* has to be performed with each sample testing (pages 29 to 32).

For the interpretation of the Assay results use only the melting curve analysis and ignore the Ct values (amplification curve).

The temperature of specific and potentially unspecific peaks depends on the used Real-Time PCR instruments. In the following sections examples are presented using the Roche LightCycler®96. Here, the specific peak of the Internal Extraction Control is located at approximately 90°C (Fig. 5, page 41, blue melting curves). It is important to calibrate other Real-Time PCR instruments for the specific temperature of the Internal Extraction Control peak (see part ‘General Requirements’ page 36).

The specific peak can vary in height (part 2 of Fig. 5, page 41; pink melting curves). In some cases, eluates of the samples can show one or two unspecific peaks (part 1 of Fig. 5, page 41, red and green melting curves). In all cases, a distinct peak must show up in the specific temperature range of the Internal Extraction Control (e.g., part 1 of Fig. 5, page 41, blue, red and green melting curves) for valid results.

Absence of a peak in the specific temperature range indicates a negative result of the Internal Extraction Control assay. In this case, the results are invalid and the extraction has to be repeated.

Exceptional case: In case of a specific signal in the sample test and absence of a peak in the Internal Extraction Control, the result of the sample is valid. In this case all PCR controls must perform as expected.

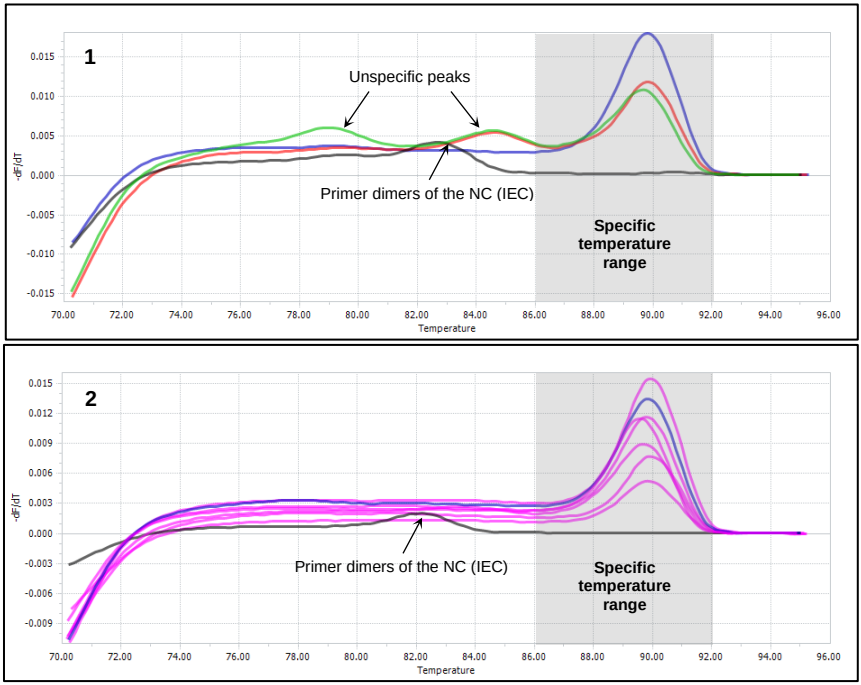


Fig. 5: Melting curve analysis (Roche LightCycler® 96) of a negative reference Internal Extraction Control (NC (IEC), black curve) and eluates of samples showing different peaks (coloured curves) in assay *MA Control*. The blue sample curve shows a specific peak (90°C) of the Internal Extraction Control. Part 1: The red sample curve shows the specific peak and an unspecific peak (85°C). The green sample curve shows three peaks (specific peak at 90°C and two unspecific peaks at 85°C and 79°C). Part 2: The pink curves show the variety in height of the specific peak. All results are valid.

Schematic Examples for the Interpretation of Real-Time PCR Results:

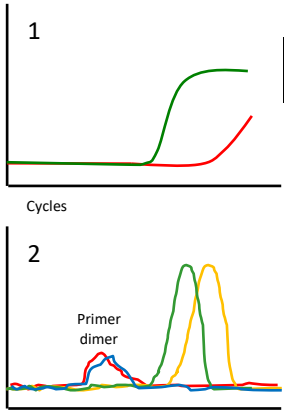
Examples (A to H, pages 42 to 44) of Real-Time PCRs are shown in a schematic modus of amplification curves (1, upper image) and melting curves (2, lower image). Absolute and relative T_m values can vary among different Real-Time PCR systems. On the right hand side the interpretation of the results is given in tables and text. The colour code in the table corresponds to the curves in the images. For *MA Control* (IEC and NC IEC) only the melting curve analysis is shown.

In this illustration it is understood that the positive PCR controls (P1 and P2) indicate full functioning of the assay.

Legend to pages 42 to 44:

Sample: Mastermix Assay Bacteria (*MA Bac*) or Mastermix Assay Yeasts (*MA Yeasts*) – green curve; **NC (sample)**: Negative PCR controls – red curve (*MA Bac / MA Yeasts*); **IEC**: Mastermix Assay Control (*MA Control*, Internal Extraction Control) – yellow curve; **NC (IEC)**: Negative reference Internal Extraction Control – blue curve (*MA Control*); **Pathogen present?**: **+** means a true positive result, **-** means a true negative result, **?** means that the result is unclear. **Figures: 1** Amplification curves; **2** Melting curve analysis.

A



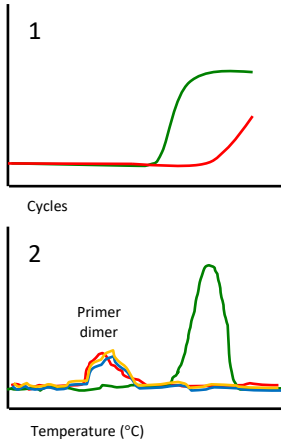
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	-	+

True positive result

The Internal Extraction Control (IEC) appears at the expected value. The reference Internal Extraction Control (NC IEC) is negative as expected. The sample is positive in the melting curve analysis and the negative PCR controls (NC´s) do not show a signal (besides primer dimers).

The positive sample result is valid.

B



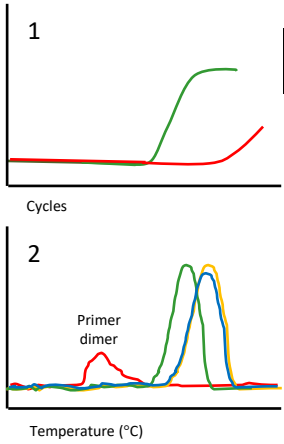
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	-	-	+

True positive result

The sample is positive in the melting curve analysis. The Internal Extraction Control (IEC) lacks a signal in the melting curve, because the *Control DNA* was not added in the extraction process or the Internal Extraction Control (IEC) PCR setup was incorrect. The negative controls (NC´s) do not show a signal (besides primer dimers).

In this case the positive sample result is valid.

C



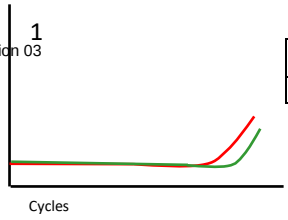
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	+	+

True positive result

The sample is positive in the melting curve analysis and the negative control (NC sample) does not show a signal (besides primer dimers). The Internal Extraction Control (IEC) is correct, but the reference Internal Extraction Control (NC IEC) shows a signal, indicating contamination in the *MA Control*. The positive result of the sample is correct.

In this case the positive sample result is valid.

D



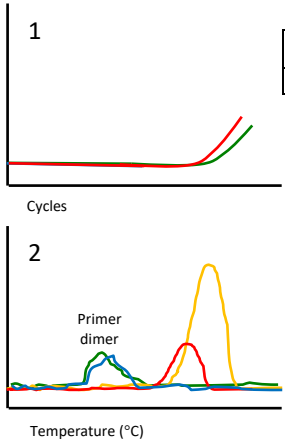
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	-	-

True negative result

The Internal Extraction Control (IEC) is at the expected value. The sample, negative control (NC) and reference Internal Extraction Control (NC IEC) lack a peak in the melting curve analysis (only primer dimers). Hence, pathogens are not present or below the detection limit.

The negative sample result is valid.

E



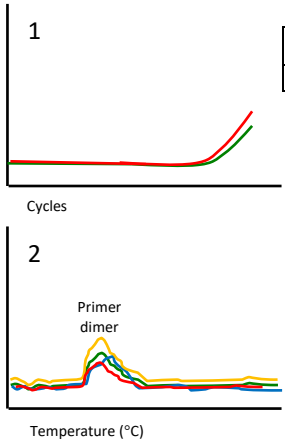
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	+	+	-	-

True negative result

The Internal Extraction Control (IEC) is regular and the sample lacks a signal. Despite a signal in the PCR negative control (NC sample), which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit.

The negative sample result is valid.

F



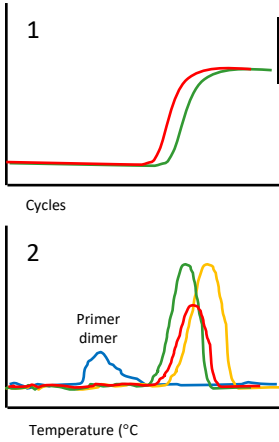
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	-	-	?

Invalid result

The PCR negative control (NC) and reference Internal Extraction Control (NC IEC) do not show a signal (besides primer dimers). The sample and the Internal Extraction Control (IEC) lack a signal (besides primer dimers) the latter indicating a failure in DNA extraction or PCR inhibition.

In such a case, the results are invalid and extraction has to be repeated.

G



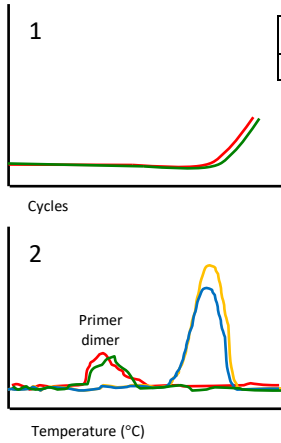
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	+	+	-	?

False positive result

The Internal Extraction Control (IEC) is regular and the reference Internal Extraction Control (NC IEC) does not show a signal (besides primer dimers). However the PCR negative control (NC sample) shows a signal, indicating contamination. It is not clear whether the signal of the sample comes from contamination or from a pathogen in the sample.

The PCR has to be repeated with more care to avoid contamination.

H



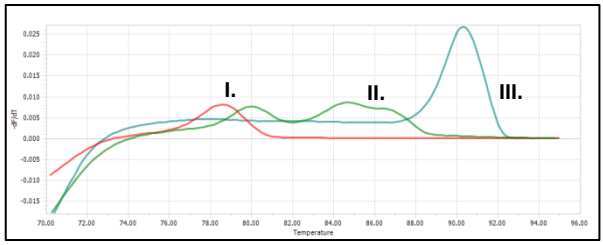
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	+	?

Invalid result

The sample and the negative control (NC sample) do not show a signal (besides primer dimers). The Internal Extraction Control (IEC) is regular, but the reference Internal Extraction Control (NC IEC) shows a signal, indicating contamination in the *MA Control*. It is not clear whether the signal of the Internal Extraction Control (IEC) comes from contamination in the PCR mix or the extraction performed correctly.

The assay Internal Extraction Control (*MA Control*) has to be repeated with more care to avoid contamination.

An example for a image of melting curve analysis (*MA Bac*):



Melting curve analysis (Roche LightCycler® 96) of a negative PCR control (I.), an eluate of a negative sample showing peaks of amplified traces of human DNA (II.) and a positive PCR control showing a specific peak (III.).

Supplementary Information

Troubleshooting

This guide may help solve problems that may arise. For further support:
Phone: +49(0)421 69 61 62 0 • **E-Mail:** support@molzym.com

Observation	Possible cause	Comments/suggestions
Weakly visible bands on agarose gel	• *DNA staining solution* (*DS*) not sufficient	The *DNA staining solution (DS)* added in the mastermix could be too weak to stain the bands sufficiently. In this case, re-stain the DNA in the gel to increase visibility of the bands. For example, use ethidium bromide according to manufacturer's instructions.
Strong human DNA background in gel electrophoresis or Real-Time PCR	• Buffer *CM* not added • Buffer *DB1* not added • *MolDNase B* not added	Eluates usually contain traces of human DNA co-eluted with bacterial/fungal DNA (Fig. 3 and Fig. 4, pages 33 to 34). If the extraction has not been performed according to the protocol, increased amounts of human DNA can be the result, which negatively influences the PCR reaction. Ensure that buffer *CM* has been added to lyse human cells. Accordingly, addition of buffer *DB1* and *MolDNase B* is obligate.
	• *MolDNase B* volume too low	Ensure that *MolDNase B* vial are briefly centrifuged before use. Make sure that the enzymes are not frozen when used.
	• *MolDNase B* incorrect usage conditions	Keep the *MolDNase B* vial chilled, because warming may reduce enzyme activity and hence human DNA background may remain high.
	• Solutions not mixed properly	It is important that solutions are thoroughly mixed after addition of buffers. Follow instructions for vortexing.
No pathogen DNA detectable in spiking tests with *SU* buffer	• Insufficient lysis	Make sure that *BugLysis* and *ß-mercaptoethanol* have been added. Ensure that the *Proteinase K* treatment has been done. Ensure that all enzyme vials are briefly centrifuged before use. Make sure that the enzymes are not frozen when used.
	• PCR inhibition	Run the Internal Extraction Control assay (*MA Control*) for testing for proper DNA extraction. Inhibitors like ethanol are co-eluted. The result of *MA Control* was negative. Repeat the extraction.
	• Insufficient homogenisation	If the pellet at steps 4 and 7 (page 24) is not homogenised, pathogen cells may be included in the debris and not reached by lytic enzymes. Follow the instructions.
	• Pathogen load too low (below limit of detection)	Check the load of the pathogen by plating and increase the titre for inoculation.
	• Loss of nucleic acids during purification	Ensure that buffer *AB* has been added to and mixed with the lysate (step 11, page 25). Accordingly, make sure that the column has been washed with buffer WB (step 13, page 25).

	• Loss of nucleic acids during the storage of the eluate	Store the eluted DNA at +4 to +12°C if analysed within 48h or freeze at -15 to -25°C until further use. Avoid frequent freeze-thaw cycles, because this may result in a decay of the eluted DNA (in particular at low DNA concentrations).
	• Wrong elution conditions	Make sure to elute with supplied heated buffer ES (70°C; step 16, page 25). This increases the DNA yield significantly.
No signal in PCR	• *MolTaq 16S/18S* not added • DS not added • *MA Bac*, *MA Yeasts* or *MA Control* not added • *H_2O* not added	Make sure that all reagents of the PCR-ready mastermixes have been added. Make sure that the *MolTaq 16S/18S* is not frozen when pipetting.
False positive PCR result (signal in negative PCR control)	• Cross contamination • Contamination during handling	Principally, work under UV-irradiated workstations. Avoid the generation of aerosols by careful pipetting. Open vials and tubes only shortly for pipetting and close again immediately thereafter. Frequently change gloves. UV-irradiate the workstation at the end of handling a series of samples. Prepare mastermixes and handle DNA samples under different UV workstations (page 19). Use DNA-free pipette tips and other plastics only as recommended (page 9).
False negative PCR result (no signal in Assay *MA Control*, Internal Extraction Control)	• PCR inhibitors co-eluted	Check whether *Proteinase K* treatment has been performed during DNA preparation and vials are briefly centrifuged before use. Make sure that all washing steps of the procedure have been followed. Optionally, after buffer *WS* washing (step 14, page 25), centrifuge for another 1min to avoid ethanol carryover to the eluate.
	• Mistakes in the extraction	Make sure that the *Control DNA* was added to buffer *RP* before using the buffer (steps 8 to 9, page 24).

References

Cayo R, Fehlberg LCC, Carvalhaes CG, Nicoletti AG, Gales AC (**2014**) Molecular diagnosis contributing for multi-drug resistant infection control. Curr Treatment Opt Inf Dis **6**, 17-39.

Chang SS, Hsieh WH, Liu TS, Lee SH, Wang CH, Chou HC, Yeo YH, Tseng CP, Lee CC (**2013**) Multiplex PCR system for rapid detection of pathogens in patients with presumed sepsis – a systemic review and meta-analysis. PLOS One **8**, e62323. doi:10.1371/journal.pone.0062323.

Chang YT, Wang HC, Wang MC, Wu AB, Sung JM, Sun HS, Su IJ, Kan WC, Chien CC, Hwang JC, Wang HY, Tseng CC, Wu CJ (**2014**) Rapid identification of bacteria and Candida pathogens in peritoneal dialysis effluent from patients with peritoneal dialysis-related peritonitis using multilocus PCR coupled with electrospray ionization mass spectrometry. J Clin Microbiol **52**, doi: 10.1128/JCM.03106-13.

Disqué C, Sakka SG, Wellinghausen N (**2010**) Klinische Evaluation von SepsiTest™ zum universellen PCR-Nachweis von bakteriellen Erregern in Vollblut. Der Mikrobiologe Heft **1**, Februar 2010, 13-16.

Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L., Rosenblatt J.E., Cockerill F.R. and Smith T.F. (**2006**) Real-Time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev*. 19: 165-256

Gebert S., Siegel D., Wellinghausen N. (**2008**) Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis. *J. Infect*.: doi:10.1016/j.jinf.2008.07.013.

Grif K, Heller W., Prodinger M, Lechleitner K, Lass-Flörl C, and Orth D (2012) Improvement of Detection of Bacterial Pathogens in Normally Sterile Body Sites with a Focus on Orthopedic Samples by Use of a Commercial 16S rRNA Broad-Range PCR and Sequence Analysis. J Clin Microbiol **50**, 2250-2254

Haag H, Locher F, Nolte O (**2013**) Molecular diagnosis of microbial aetiologies using SepsiTest™ in the daily routine of a diagnostic laboratory. Diag Microbiol Inf Dis **76**, 413-418.

Handschur M., Karlic H., Hertel C., Pfeilstöcker M., Haslberger A.G. (**2008**) Pre-analytic removal of human DNA eliminates false signals in general 16S rDNA PCR monitoring of bacterial pathogens in blood. *Comp. Immun. Microbiol. Infect. Dis*.: doi:10.1016/j.cimid.2007.10.005.

Horz H.-P., Scheer S., Huenger, F., Vianna M., Conrads G. (**2007**) Selective isolation of bacterial DNA from human clinical specimens. *J. Microbiol. Meth*. 72: 98-102.

Igressa A, Defosse J, Disqué C, Wappler F, Sakka SG (**2014**) Usefulness of broad-range PCR plus sequencing for the diagnosis of bacteremia due to a lung abscess. Int J Infect Dis, DOI: 10.1016/j.ijid.2014.04.018.

Kabir MA, Ahmad Z (**2013**) Candida infections and their prevention. ISRN Preventive Medicine, vol. 2013, Article ID 763628, 13 pages, 2013. doi:10.5402/2013/763628.

Kothari A, Morgan M, Haake D (**2014**) Emerging technologies for rapid identification of bloodstream pathogens. Clin Inf Dis, doi: 10.1093/cid/ciu292.

Kühn C, Orszag P, Burgwitz K, Marsch G, Stumpp N, Stiesch M, Haverich A (2013) Microbial adhesion on membrane oxygenators in patients requiring extracorporeal life support detected by a universal rDNA PCR test. ASAIO Journal **59**, 368-373.

Kühn C., Disqué C., Mühl H., Orszag P., Stiesch M., Haverich A. (**2011**) Evaluation of commercial universal rRNA gene PCR plus sequencing tests for identification of bacteria and fungi associated with infectious endocarditis. J Clin Microbiol **49**, 2919-2923.

Lebovitz EE, Burbelo PD (**2013**) Commercial multiplex technologies for the microbiological diagnosis of sepsis. Mol Diagn Ther **17**, 221-231.

Liste der vom Robert-Koch-Institut geprüften und anerkannten Desinfektionsmittel und -verfahren (**2007**) Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz 50:1335–1356

Loonen AJM, Wolffs PFG, Bruggeman CA, van der Brule AJC (**2014**) Developments for improved diagnosis of bacterial bloodstream infections. Eur J Clin Microbiol Inf Dis, doi: 10.1007/s10096-014-2153-4.

Marsch G, Orszag P, Mashaqi B, Kuehn C, Haverich A (**2015**) Antibiotic therapy following polymerase chain reaction diagnosis of infective endocarditis: a single centre experience. Interactive CardioVascular and Thoracic Surgery 1–5. DOI:10.1093/icvts/ivv006.

Meyer T, Franke G, Polywka SKA, Lütgehetmann M, Gbadamosi J, Magnus T, Aepfelbacher M (**2014**) Improved Detection of Bacterial Central Nervous System Infections by Use of a Broad-Range PCR Assay J. Clin. Microbiol **52**(5):1751. DOI: 10.1128/JCM.00469-14.

Mühl H., Kochem A.-J., Disqué C., Sakka S.G. (**2010**) Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16S rDNA real-time polymerase chain reaction detection of bacterial pathogens in blood. *Diagn. Microbiol. Infect. Dis*. 66: 41-49.

Nieman AE, Rozemeijer W, de Jong E, Beishuizen B, van Agtmael MA, Savelkoul PHM, Schade RP (**2010**) Molecular detection of bacterial bloodstream infections: validation of the SepsiTest™ assay. Ned Tijdschr Med Microbiol 1**8** Supplement, Abstract P048, 109.

Oliva A, Belvisi V, Iannetta M, Andreoni C, Mascellino MT, Miriam Lichtner M, Vullo V, Mastroianni CM (**2010**) Pacemaker lead endocarditis due to multidrug-resistant Corynebacterium striatum detected with sonication of the device. J Clin Microbiol **48**, 4669-4671.

Orszag P, Disqué C, Keim S, Lorenz MG, Wiesner O, Hadem J, Stiesch M, Haverich A, Kühn C (**2013**) Monitoring of patients supported by extracorporeal membrane oxygenation for systemic infections by broad-range rRNA gene PCR amplification and sequence analysis. J Clin Microbiol **52**, 307-311.

Pletz MW, Wellinghausen N, Welte T (**2011**) Will polymerase chain reaction (PCR)-based diagnostics improve outcome in septic patients? A clinical view. Intensive Care Med **37**, 1069–1076.

Rogina P, Skvarc M, Stubljar D, Kofol R, Kaasch A (**2014**) Diagnostic utility of broad range bacterial 16S rRNA gene PCR with degradation of human and free bacterial DNA in bloodstream infection is more sensitive than an in-house developed PCR without degradation of human and free bacterial DNA. Mediators of Inflammation, doi.org/10.1155/2014/108592.

Roth A., Mauch H. and Göbel U.B. (**2001**) Quality standards for microbiological diagnostic techniques for infectious diseases - 1. Nucleic acid amplification techniques. *Urban & Fischer Verlag*, München-Jena

Sakka SG, Kochem AJ, Disqué C, Wellinghausen N (**2009**) Blood infection diagnosis by 16S rDNA broad-spectrum polymerase chain reaction: the relationship between antibiotic treatment and bacterial DNA load. Anesthesia Analgesia **109**, 1707-1708.

Schaub N, Frei R, Müller C (**2011**) Addressing unmet clinical needs in the early diagnosis of sepsis. Swiss Med Wkly. **141** (in press); doi:10.4414/smw.2011.13244

Schreiber J, Nierhaus A, Braune SA, de Heer G, Kluge S (**2013**) Comparison of three different commercial PCR assays for the detection of pathogens in critically ill sepsis patients. Med Klin Intensivmed Notfmed doi 10.1007/s00063-013-0227-1.

Schukfeh N, Doerner JM, Heintschel von Heinegg E, Steinmann J, Metzelder ML, Kathemann S, Hoyer PF, Paul A, Gerner P (**2014**) Spectrum of pathogens in native liver, bile, and blood during pediatric liver transplantation. Ped Transplantation **18**, 266–271.

Shin JH, Sharaan M, Wu J, Cui M, Zhang DY, Tang YW (2013) Molecular bacteriology mycobacteriology. In: Cheng L, Zhang DY, Eble JN (eds.), Molecular Genetic Pathology. Springer Science and Business Media, New York. doi 10.1007/978-1-4614-4800-6_26.

Skvarc M, Stubljar D, Rogina P, Kaasch AJ (**2013**) Non-culture based methods to diagnose bloodstream infections: does it work? Eur J Microbiol Immunol **3**, 97–104.

Stubljar D, Kopitar AN, Groselj-Grenc M, Suhadolc K, Fabjan T, Skvarc M (**2015**) Diagnostic accuracy of presepsin (sCD14-ST) to predict bacterial infection measured in cerebrospinal fluid in children with suspected bacterial meningitis/ventriculitis. J Clin Microbiol; doi:10.1128/JCM.03052-14.

Stubljar D, Skvarc M (**2014**) Expression of CD64 on neutrophils can be used to predict the severity of bloodstream infection before broad range 16S rRNA PCR. Folia Microbiologica; DOI **10**.1007/s12223-014-0346-y.

Valencia-Shelton F, Loeffelholz M (**2014**) Nonculture techniques for the detection of bacteremia and fungemia. Future Microbiol 9, 543-559.

Vollzugshilfe zur Entsorgung von Abfällen aus Einrichtungen des Gesundheitsdienstes vom **01.01.2021**, Robert-Koch-Institut

von Loewenich FD, Geißdörfer W, Disqué C, Matten J, Schett G, Sakka SG, Bogdan C (**2010**) Detection of "Candidatus Neoehrlichia mikurensis" in two patients with severe febrile illnesses: evidence for a european sequence variant. J Clin Microbiol **48**, 2630-2635.

Wellinghausen N., Kochem A.J., Disqué C., Mühl H., Gebert S., Winter J., Matten J. and Sakka S.G. (**2009**) Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. J. Clin. Microbiol. **47**: 2759-2765

Wu CJ, Chen YP, Wang HC, Su IJ, Ko WC, Chen JS, Cheng CN, Lee NY, Sun HS, Chi CY, Chen TY (**2013**) Identification of fungal pathogens from clinical specimens using multi-locus PCR coupled with electrospray ionization mass spectrometry. Diagn Microbiol Infect Dis 7**8**, 141-143.

Tradenames

Tradename	Factory
ABI 3730XL® and ABI Prism310®	Applied Biosystems
ABI 7500 Fast®	Life Technologies
Add-On 10	Molyzm
BigDye®	Applied Biosystems
BioBall® MultiShot 550 KBE	bioMérieux
Biosphere®plus	Sarstedt
CFX96™	BioRad
DNA Engine Opticon®	BioRad
DNA/RNA- ExitusPlus™,	AppliChem
GuardOne® Werkbank	Starlab
LightCycler® 1.5, 2.0, 96, 480 and Nano	Roche
Mastercycler®	Eppendorf
Meliseptol® New Formula	B. Braun
Micro-Dx™	Molzym
Mx3000P® and Mx3005P®	Stratagene
PCR strip of 4, 200 µl, Biosphere® plus	Sarstedt
peqStar 96Q	peqlab
QiaQuick®	Qiagen
Reliant®	Lonza
RipSeq®	Pathogenomix
Rotor-Gene®	Qiagen
SelectNA™*plus*	Molzym
SepsiTest™-UMD	Molzym
S-Monovette®	Sarstedt
SYBR® Green1	Invitrogen
UMD Tubes	Molzym

Technical Support

If you have questions please call us.

Our hotline: +49(0)421 69 61 62 0 • **E-Mail:** support@molzym.com

Material safety data sheets are available on request.

Also visit Molzym's homepages for further information:
http://www.molzym.com

Order Information

Product	Contents and Application	Cat. No.
SepsiTest™-UMD	**24** reactions	U-010-024
(CE IVD)	**48** reactions	U-010-048
	DNA isolation, PCR detection, manual	

Other Products

Product	Contents and Application	Cat. No.
UMD Tubes	**20 vials**	Z-801-020
Sample storage (research use only)	Storage of blood and other primary body fluids. Sample volume: 0.4 to 2 ml	
Add-On 10	**24** reactions	U-120-024
(CE IVD)	**48** reactions	U-120-048
	Add on kit for **SepsiTest™-UMD.** Blood and other primary body fluid samples >1 to 10 ml	
Automated Pathogen DNA Extraction and PCR Analysis		
Micro-Dx™	**24** reactions	U-200-024
(CE IVD)	**48** reactions	U-200-048
	DNA isolation, PCR detection, manual	
SelectNA™plus necessary for processing of the *Micro-Dx™*.	**Instrument** for Automated Pathogen DNA Extraction for 1 to 12 samples of body fluids, swabs and tissues.	D-400-001

Order Hotline:

Tel.: +49(0)421 69 61 62 0 • **Fax:** +49(0)421 69 61 62 11 • **E-Mail:** order@molzym.com

Contact

Molzym GmbH & Co. KG
Mary-Astell Str. 10
28359 Bremen, Germany

Tel.: +49(0)421 69 61 62 0 • **Fax:** +49(0)421 69 61 62 11
E-Mail: info@molzym.com • **Web:** www.molzym.com